Tissue engineering for reconstructing the central dopaminergic nigro-striatal pathway in Parkinson's disease: Cutting edge cell culture studies

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Table of Contents

Summary

Although neurotransplantation of primary fetal cells into the striatum of patients with Parkinson's disease (PD) has been reported to be effective, poor clinical outcome and severe side effects lower clinical long-term results. A major drawback of cell replacement therapies in PD is the low cell survival and lacking regeneration of the neuronal circuitries due to the *ectopic* transplantation of cells into the host striatum. More anatomic and functional integration could potentially be reached by an *orthotopic* cell transplantation into their natural position within the rostral mesencephalon at the site of the *substantia nigra*, where dopaminergic cells get lost in PD. The aim of the thesis was to provide the scientific basis for the use of injectable bioscaffols containing chemo-attractants promoting cell survival, differentiation and axo-dendritic outgrowth of dopaminergic cells. With the so called "bridging" transplantation technology an artificial axon pathway between the *substantia nigra* and the striatum with targeted nigro-striatal re-innervation should be generated. Thereby, the central dopaminergic nigro-striatal pathway would be reconstructed enabling a fully integration of grafted neurons into the basal ganglia circuitries.

The main focus of the thesis was to explore the influence of bioscaffolds on cell survival and morphology of dopaminergic neurons *in vitro*. The investigations included isolation of primary fetal mesencephalic cells and fetal mesencephalic neural stem cells (NSCs) from embryonic (E14) mouse brain and their culture on ECM compounds and starPEG-heparin hydrogels. Initial characterizations of the gels showed separate as well as simultaneous immobilization and release of growth factors demonstrating that hydrogels could serve as an efficient storage and delivery system for growth factors. The axo-dendritic outgrowth of dopaminergic cells including primary branching, total branching and neurite elongation; cell survival studies; cell type analysis and cell migration were analyzed by immunostaining.

Both cell sources showed distinct growth properties depending on the stiffness of the gel material and the presence of biomolecules with increased cell survival by the presence of RGD and FGF-2 in the hydrogel independent of network characteristic. Moreover, the presence of RGD on hydrogels was found to initiate differentiation of NSCs, whereas FGF-2 bound to hydrogels was shown to promote the viability of undifferentiated cells. Additionally, survival and axo-dendritic outgrowth

of dopaminergic cells were observed to be affected by the gel properties: RGD or FGF-2 modification of hydrogels with intermediate network density showed the best results for dopaminergic growth. With the addition of GDNF to hydrogels the total amount of cells decreased strongly by an equal quantity of dead cells compared to FGF-2 bound hydrogels. Furthermore, differential effects were found for the survival of different brain cells depending on the growth factor which is loaded. GDNF was found to increase the survival of astrocytes, whereas FGF-2 bound to gels stimulated the viability of oligodendrocyte precursor cells. No differential effects were found for the survival of NSCs and mature neuronal cells on GDNF or FGF-2 bound gels.

By showing the penetration of primary fetal mesencephalic cells expressing MMPs as endogenous endopeptidases into MMP-cleavable hydrogels, the potential biodegradability of the starPEG-heparin hydrogels was demonstrated.

Together the findings provide the *in vitro* proof-of-principle data for combining dopaminergic neurons or predopaminergic NSCs with biomaterials for reconstructing the central dopaminergic nigro-striatal pathway by the “bridging” transplantation strategy as an alternative transplantation approach in PD. Further studies should focus on three-dimensional cell culture studies using starPEG-heparin hydrogels with cleavable peptide sequences and their functionalization with gradients of axon guidance molecules to selectively promote dopaminergic outgrowth.

1. Introduction

1.1 Tissue engineering and regenerative medicine

The generation of tissue, organs or even complex organisms was for a long time a vision of mankind throughout the history of medicine. Until today a lot of pioneering work is done to generate or regenerate tissue and organs. First bioengineered products are applied in the clinical reality and much more are in preclinical stage. The terms *tissue engineering* and *regenerative medicine* are nowadays considered as an approach to generate complex tissue and organs from simple parts. While tissue engineering more address the technical concept of reconstructing tissue and organs with the help of cells, biomolecules and scaffolds, the term regenerative medicine is more focused on the support of self healing abilities and on the use of stem cells. In this young field of biotechnology various scientific disciplines such as biochemistry, cell biology, pharmacology, material science, engineering and clinical disciplines are closely connected and all tracing the same intention of regenerating damaged tissue by implanting cells in combination with scaffolds. On this way several challenges need to be met concerning scientific, technological, clinical, ethical, and also social issues.

Fundamental for tissue engineering is a successful *in vitro* cultivation of cells. A breakthrough was reached by Ross G. Harrison in 1907 demonstrating active growth of nerve fibers *in vitro*. He was a pioneer who first successfully overcame basic culture problems and created a technique for other scientists. In 1935, Alexis Carrel and Charles Lindbergh published the book “The Culture of Whole Organs” describing the *ex vivo* culture technique for organs including the use of a perfusion pump [1]. In the early and mid 20th century lots of successful transplantations of many organs followed. However, organ transplantation as a long-term solution is a life-saving therapy with many clinical problems concerning a limited supply of donor organs/tissue, the possibility of rejection and the use for long-term immunosuppression. Tissue engineering strategies will ideally overcome these difficulties.

The first synthetic polymeric material based on poly(vinyl chloride) were grafted together with endothelial cells in dogs to replace aortic segments [2]. A similar

approach by William Chick and his colleagues involved seeding pancreatic beta cells on a semipermeable membrane [3]. Thus, living and artificial components were combined and used to successfully treat diabetes in rats [4] and dogs [5]. In the early 1970s W.T. Green studied cartilage formation *in vitro* and *in vivo* using a chondrocyte culture technique in combination with specifically tailored scaffolds [6]. Despite of his inability to generate new cartilage, he established the theoretical and practical concept of combining cells with scaffolds. With the first *in vitro* cell-scaffold approach described by John Burke and Ioannis Yannas in 1981 the generation of skin by a culture of dermal fibroblasts and keratinocytes on a collagen and silicon based material was an innovation in regeneration of burn wounds [7]. With this dermal template they could encourage the ingrowth of native skin and vessels from the surrounding skin. A similar approach used *in vitro* expanded autologous dermal fibroblast and epidermal keratinocytes seeded on a collagen matrix to generate a full thickness skin graft [8]. Within the scientific community the term and the field tissue engineering was on the way to become established. Publication of an article by Joseph Vacanti and Robert Langer in Science in 1993 was a key point in tissue engineering introducing the field to a broader scientific community and describing the new technology with all critical areas [9]. It is referenced as the beginning of this new biomedical discipline.

As the use of extracellular matrix (ECM) has led to a number of exciting advances in tissue engineering, the use of biological scaffolds deserved closer attention. Now, ECM-based strategies with decellularized matrix products engineered from various parts of the human body were combined with autologous cells to regenerate patient-specific tissues [10, 11]. For this strategy the supply of available materials is also limited relative to solutions using synthetic biomaterials. Therefore, the development of functional synthetic extracellular matrices may further reduce the need for donor tissue.

Essential for tissue engineering is beside generation of a proper bioscaffold a well-characterized, easy accessible cell population. In addition ideal growth factor or conditioning regimes for differentiation and maintenance of differentiated stage are required. While the use of embryonic stem (ES) cells for tissue engineering strategies is hampered by ethical concerns as well as the fact that ES cells are more prone to favor tumor or teratoma progression, the identification and the use of other cell

sources like adult stem cells and induced pluripotent stem cells is a promising strategy. Furthermore, the use of autologous tissue for transplantation eliminates the need of immunosuppression. With their successful cultivation and their capability to differentiate into different cell types they are a prospective cell source for cell replacement therapies.

1.2 Restorative treatments for Parkinson's disease

1.2.1 Main aspects of Parkinson's disease and available therapies

Parkinson's disease (PD) is a chronic, slow progressive neurodegenerative disorder of the central nervous system. Due to the selective loss of dopaminergic (DAergic) neurons within the *substantia nigra* in the midbrain the dopamine release in the target region – the striatum – is strongly reduced. Under healthy conditions DAergic cells project their axons from the *substantia nigra* via the nigro-striatal pathway to the striatum, where an extensively branching takes place and the neurotransmitter dopamine is released. The death of these cell type results in the degeneration of axonal projections into the striatum, and thus the nigro-striatal pathway is destroyed. Several motor symptoms, such as rigidity, tremor and bradykinesia as well as non-motor symptoms like disabilities of speech, cognition, mood, and behavior are a result of the destroyed nigro-striatal pathway in PD patients. The reasons for this continuous and selective loss of DAergic neurons still remain unclear. The absence of enzymes, neurotransmitters, trophic factors or the existence of toxic factors or physical injuries may play a role at the onset and the progression of PD.

Current therapies for PD are primarily based on pharmacological replacement of lost striatal dopamine by Levodopa administration, which subsequently improves motor symptoms [12, 13]. These approaches remarkably moderate PD symptoms, especially in early stages of the disease [12, 13]. At progressive stages of disorder the efficacy of these therapy declines and many patients develop drug-induced dyskinesia. Therefore, electrical deep brain stimulation in the Nucleus subthalamicus was developed [12, 14, 15]. This treatment alleviates only some of the symptoms resulting from a partial normalization of the neuronal activity. So far, these therapies are only symptomatic treatments of the nigro-striatal deficit associated with PD.

Hence, the need for an improved long-term treatment is evident. A possible strategy is to restore the lost neuronal subtype by cell transplantation.

1.2.2 Stem cell-based cell sources for cell replacement therapies in PD

Neural transplantation in PD is based on a well-defined biological mechanism: Recovery of function by restoration of dopaminergic transmission in the striatum. Possible cell sources for cell replacement therapies in PD are fetal primary tissue, fetal neural stem cells (NSCs), embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, adult brain-derived stem cells and adult multipotent stem cells [16].

NSCs are multipotent stem cells derived from the nervous system with regenerative capacity. They can differentiate into all three major cell lineages of the nervous system: astrocytes, oligodendrocytes and neurons [17-20]. An initial study for *in vitro* culturing of NSCs showed that fibroblast growth factor-2 (FGF-2) stimulates the proliferation of rat neuronal precursor cells [21]. Furthermore, the induction of the growth of striatal precursors retaining their capability to differentiate into all major cell lineages has been demonstrated [22]. Since these studies two mitogens (EGF and FGF-2) were used for the *in vitro* proliferation of fetal NSCs [23]. For generating DAergic neurons, the differentiation of mesencephalic NSCs *in vitro* by specific growth factors or cytokines activating the expression of transcription factors, such as Nurr1 and Pitx3, is necessary [17]. For the expansion of mesencephalic NSCs from rodents and human over a long-term period and with stable growth behavior and apoptosis rate a level of 3 % oxygen is necessary [24-27]. However, long-term survival and phenotype stability of DAergic neurons generated from fetal NSCs after transplantation in animal models of PD still have to be demonstrated. The low tumorigenic formation of fetal NSCs in comparison to ES cells, immortalized cell sources and iPS cells seems to be a major advantage, whereas their low expansion and differentiation as well as ethical concerns limit their use for cell replacement therapies.

Human ES cells with their characteristics of self-renewal and pluripotency differentiating in any of the three germ layers seem to be a reliable cell source. Prior to transplantation into animals models of PD ES cells were differentiated *in vitro* into neural precursors [28] or DA neurons [29, 30] showing in some cases a partially functional recovery. Using ES cells in cell replacement therapy in PD, there is the

problem of controlling cell growth and differentiation. In most studies using ES cells for transplantation brain tumor or teratoma formation and a low graft survival were reported [31-34].

The pioneering work of Takahashi and Yamanaka in 2006 generating iPS cells from mouse fibroblast afford a new opportunity to generate cells with characteristics of ES cells [35]. Neurons derived from reprogrammed mouse fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease [36]. As an autologous cell source, patient specific iPS cells exhibit an interesting tool for regenerative cell therapy approaches due to no ethical concerns and no needed immunosuppression. Moreover, their differentiation into DA neurons *in vitro* could be shown recently [37]. The growth of differentiated Parkinson patient-derived iPS cells in the adult rodent brain without signs of neurodegeneration and a reduction of motor asymmetry in Parkinsonian rats were reported [38]. For the generation of iPS cells most of the reprogramming strategies are using viral transfection of putative oncogenes, which my cause cancer by integrating into the genome. The use of adenovirus [39], plasmids [40] and recombinant proteins [41], [42] could be an alternative way for reprogramming. However, a lot of hurdles have to be taken before the iPS strategy can be used for clinical applications.

1.2.3 Cell transplantation strategies for Parkinson's disease

Clinical trials of neurotransplantation using human mesencephalic DAergic neurons provided the proof-of-principle for dopaminergic cell replacement strategies [43, 44]. The main transplantation studies have focused on re-innervating the striatum by *ectopic* placement of DAergic grafts (Figure 1A). PET scans and postmortem studies showed that DAergic neurons implanted into the striatum of patients with PD survive and re-innervate the striatum. Although *ectopic* transplantation has demonstrated a successful method of integration of implanted DAergic neurons into the host striatum, only a subset of younger patients showed clinical benefits [43]. Furthermore, in another double-blinded, placebo controlled (sham surgery) trial of bilateral fetal tissue transplantation fifty-six percent of transplanted patients suffered from severe dyskinesia after overnight withdrawal of dopaminergic medication [44]. Some patients could benefit from the transplantation of human fetal cells [34, 45] but limited tissue availability as well as ethical concerns requires alternative cellular sources.

As *ectopic* transplantation of primary fetal cells into the striatum exhibits only poor clinical outcome and severe side effects further progress had to be done in the development of an alternative transplantation strategy in PD. More anatomic and functional integration could be reached by an *orthotopic* cell transplantation into the natural position within the rostral mesencephalon (Figure 1B) [46]. Therefore, the main focus is the reconstruction of the nigro-striatal pathway to enable a fully integration of grafted neurons into the basal ganglia circuitries. The feasibility and safety of intranigral transplantation have been shown in rodents [47-50] and humans [51, 52]. Graft integration, survival and partial functional recovery have been demonstrated and allow the assumption for the existence of factors guiding targeted nerve fiber outgrowth from the *substantia nigra* to the striatum [47-50]. Recently, anatomical and functional reconstruction of the nigro-striatal DAergic pathway by intranigral transplantation of fetal cells has been demonstrated for the first time [53, 54]. With the so called “bridging” technology an artificial axonal pathway between *Substantia nigra* and striatum with targeted nigro-striatal re-innervation is generated by growth factors, other cell types or chemicals using primary DAergic cells [50, 55, 56]. One of these transplantation studies used kainic acid, an excitatory amino acid, as bridging molecule [50]. Similar results were shown using kidney tissue as bridging tissue, known to produce high amounts of glial cell line-derived neurotrophic factor (GDNF). Tyrosine hydroxylase (TH) positive fiber tracts from the *Substantia nigra* re-innervate the striatum in hemi-parkinsonian rats [55]. In both studies, a trophic environment could be triggered by the bridging materials guiding effectively transplanted neurons to send axons through the track innervating the distal striatum and thus to restore the nigro-striatal pathway. Together, a successful reconstruction of the nigro-striatal pathway in PD animal models could be shown using the “bridging” strategy. Major concerns still exist about the used bridging material. For an application in humans the development of another source of material with controlled release of neurotrophic factors, improved survival of DAergic neurons, as well as promoted growth of their axons are indispensable.

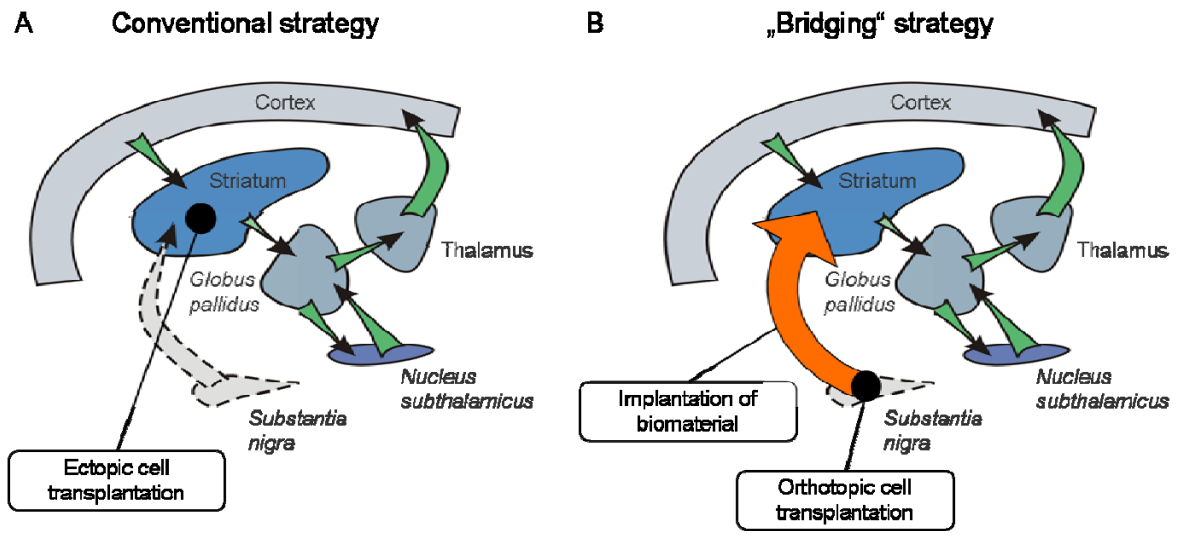

Figure 1: Cell transplantation strategies in Parkinson's disease. (A) As a conventional strategy cells are transplanted ectopically in the striatum. The dotted areas highlight the lost DAergic neurons in the *Substantia nigra* and the degenerated nigro-striatal pathway resulting in a reduced innervation of dopaminergic axons in the striatum. (B) With the "bridging" strategy cells are transplanted in the *Substantia nigra* and the nigro-striatal pathway is reconstructed by implantation of a biomaterial promoting cell survival in the *Substantia nigra* as wells as axo-dendritic outgrowth to re-innervate the striatum. Thus, a more anatomic and functional integration could be achieved (modified from [57]).

1.2.4 Potent growth factors promoting survival and axo-dendritic outgrowth of DAergic neurons

Growth factors are steroid hormones or proteins stimulating cell growth, proliferation and differentiation. They are important for regulating a variety of cellular processes. Such signaling molecules are secreted and bind to specific transmembrane receptors on the surface of their target cells. Acting as growth factors the neurotrophic factors are important for promoting growth and differentiation of neurons during development as well as phenotypic maintenance of adult mature neurons. In PD neurotrophic factors provide important therapeutic effects by promoting the survival of DAergic neurons and protecting this specific cell type after administration of dopamine neuron death inducing toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridne (MPTP) and 6-hydroxydopamine (6-OHDA). Furthermore, they are able to restore the function of moribund DAergic neurons by changing the gene expression and protein synthesis and may stimulate the dopaminergic system by affecting the ion channels leading to modifications in cell excitability for instance [58].

Neurotrophic factor are classified into several families of structurally and functionally related molecules: (1) Nerve growth factor (NGF)-superfamily; (2) glial cell line-derived neurotrophic factor (GDNF) family; (3) neurokine or neuropoietin superfamily; (4) non-neuronal growth factor-superfamily [59]. Several factors are considered as potential therapeutic molecules for therapies in PD. The survival of embryonic DAergic neurons *in vitro* and *in vivo* have been demonstrated for some neurotrophic factors as well as non-neuronal growth factors including brain-derived neurotrophic factor (BDNF) [60], neurotrophin-3 (NT-3) [61], neurotrophin-4/5 (NT-4/5) [62], GDNF [63, 64], transforming growth factor-beta (TGF-ß) [65, 66], insulin-like growth factor (IGF-1) [67], epidermal growth factor (EGF) [68] and both acidic fibroblast growth factor (aFGF/FGF-1) and basic fibroblast growth factor (bFGF/FGF-2) [69-71]. Recently, a new family of neurotrophic factors containing cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF) have been reported protecting midbrain DAergic neurons [72, 73].

Besides promoting survival of DAergic neurons axo-dendritic guidance cues could be conductive for reconstruction of the nigro-striatal pathway. For many neurotrophic factors, such as GDNF [64, 74], NT3 and BDNF [75], an axo-dendritic growth promoting effect on DAergic neurons had been shown. Recently, several other factors including Slit-1 and -2 [76]; Netrin-1 [77]; ephrin-a [78, 79] and semaphorins [80, 81] had been identified to play a role for midbrain DAergic axon pathfinding. Netrin-1 and Sonic hedgehog (Shh) act as a chemoattractant molecule for midbrain DA neurons *in vitro* [77, 82].

1.2.5 Strategies for GDNF and FGF-2 delivery to the brain

Since GDNF has protective, restorative and axonal promoting properties, this promising molecule has been studied extensively *in vitro* as well as in animal models of PD. Human GDNF gene encodes for a 134 amino acid mature protein forming a two15 kDa disulfide-linked homodimeric glycoprotein. Large size of the protein, chemical structure and fast degradation makes the entry into the CNS through the blood-brain barrier (BBB) impossible.

Different methods for the delivery of trophic factors to the CNS were applied bypassing the BBB. Although intracerebroventricular delivery of GDNF increased the

number of DA neurons in the midbrain of MPTP depleted monkeys, this method was not successful in humans [83, 84]. It is extremely likely, that the diffusion of the factor from the ventricular ependyma to the putamen through the much larger human brain is not possible.

Another delivery method into the striatum is the direct infusion of trophic factor with minipumps [85]. An advantage of this administration is the complete control of the delivered dose, but the limited diffusion of the protein resulting in a concentration gradient generated at the point of infusion and thus the use of higher concentration of neurotrophic factor is a major limitation of this delivery method. Therefore, high concentrations of the neurotrophic factor as well as pump refilling are needed.

Constant and local *in situ* expression of GDNF can be achieved by recombinant viral transduction [86]. Adenovirus (AV), adeno-associated virus (AAV) and lentivirus (LV) have been successfully tested for durable expression of GDNF in animal models of PD [87-89]. As a proof-of-principle, Bilang-Bleuel A. and coworkers have been demonstrated the prevention of DA neuron degeneration by intrastriatal injection of AV vectors expressing GDNF in 6-OHDA lesioned rats [87]. Concerns about the induction of a strong immune response directed the focus to AAV as well as LV vector systems for *in vivo* gene delivery. Both strategies showed promising results [89, 90] but the risk of tumor formation limit the application of lentiviral vectors. So far, AAV-mediated gene delivery seems to be the most powerful system, but concerns about the exact GDNF amount produced from the viral-infected neurons as well as control over the temporal gene expression still remain.

An alternative gene therapy approach to circumvent concerns about the direct injection of viral vectors into the brain is the *ex vivo* gene transfer of GDNF and encapsulated cells. The most promising cell source for *ex vivo* gene transfer are NSCs which can be differentiated into a particular neuron cell type and do not form any teratomas. The main risk for transplantation of genetically engineered cells is that the cells can be rejected by the immune system of the host. Therefore, the cells have to be encapsulated into macro- or microcapsules. The encapsulating material should allow the diffusion of nutrients from the brain to the cells and vice versa the secretion of trophic factors. An 8-week survival time of genetically modified cells producing GDNF in the striatum of 6-OHDA parkinsonian rats and protection of the dopamine system has been demonstrated [91]. A major drawback of using capsules is that the

minipores can become blogged resulting in an insufficient supply of nutrients to the cells which can cause the death of the encapsulated cells.

Another strategy for slow GDNF delivery is the use of drug-releasing biocompatible, biodegradable and non-toxic polymer-based compounds. This has been tested for microspheres made of poly(lactic-co-glycolic) acid (PLGA) polymer [92-95]. GDNF has been successfully encapsulated into PLGA microspheres and were released in a controlled manner over long time in the striatum [96, 97]. Recovery of function and induced sprouting of dopaminergic fibers have been demonstrated after implantation of GDNF-releasing microspheres in the striatum of parkinsonian rats [98] [99].

For GDNF delivery in the brain different methods are used to supply the cells with growth factor in the brain region, the striatum. This drug-delivery method will not result in a natural reconstruction of the nigro-striatal pathway and does not allow a more complete functional recovery.

FGF-2 belongs to the fibroblast growth factor family with at least 25 family members. Human FGF-2 encodes for a 146 amino acid mature protein with a 17 amino acid heparin binding site. It is an extensively studied mitogenic growth factor known to induce proliferation of neural precursors in embryonic hippocampal culture [21]. Research in studying the delivery of FGF-2 to the brain is mainly done using viral infected cells [100, 101]. Thereby, King and colleagues could show an increase in cell division *in vivo* [100]. So far, no studies have been done using other strategies such as polymer drug delivery for a FGF-2 release in animal models of Parkinson's disease.

To summarize, the polymer based delivery of GDNF is of great importance due to long-term release of factors locally at the site of implantation and compared to viral transduction it is a safer method. So far, studies only focused on GDNF delivery to the striatum without replacing lost dopaminergic cells in the substantia nigra. Combining both strategies of cell replacement in the *substantia nigra* and factor delivery spatially and temporally to the brain using polymers could show promising results for the restoration of the nigrostriatal pathway. Moreover, polymers could give structural support to cells transplanted in the *substantia nigra* and promote axo-

dendritic outgrowth towards the striatum. This could be an alternative strategy for the treatment of PD.

1.3 The extracellular matrix

1.3.1 Composition of the extracellular matrix

The extracellular part of tissues is filled by a complex network of macromolecules generating the extracellular matrix (ECM). The ECM consists of two main classes of macromolecules, which have both structural and adhesive functions: (1) Fibrous proteins, such as collagen, fibronectin, laminin and elastin, and (2) glycosaminoglycans (GAGs), which are polysaccharide chains and usually linked covently to proteins forming proteoglycans. Proteoglycan form a highly hydrated, gel-like matrix in which the fibrous proteins are embedded. Gels of polysaccharides resist compressive forces on the ECM, while collagen fibers provide strength and elastin resilience. The relative amount of the different types of matrix macromolecules and the way in which they are organized differ depending on the functional requirements of the particular tissue. Components of the ECM are produced intracellularly and secreted locally by the cells itself and are assembled into a complex structural and functional network in close association to the surface of the cell. Once thought that ECM serves mainly as a relatively inert scaffold stabilizing the physical structure of the tissue, today its known that ECM has a more complex role in native tissue, such as providing support and anchorage for cells as well as regulating intracellular signaling and dynamic behavior of the cells. Cell adhesion to ECM proteins is mediated by integrins, a class of cell surface proteins. These receptors anchor the cells to the ECM and also induce intracellular signaling events regulating cell survival, development, migration, proliferation, differentiation, survival, shape and function of the cells.

Collagen is the most abundant protein in mammals constituting 25% of the total protein mass in these animals and accounts for 90% of bone matrix protein content. A collagen α chain is about 1000 amino acids long and composed of a series of triplet Gly-Pro-X or Gly-X-Hyp sequences with glycine as every third amino

acid. As the smallest amino acid with no side chain glycine allows tight packing of three left-handed α chains to form a right-handed superhelix where the chains are hydrogen-bonded to each other. Proline and hydroxyproline stabilize the helical conformation in each α chain with its ring structure and form a helix spontaneously without any intrachain hydrogen bonding, where X can be any other amino acid. After the procollagen is secreted from the cell by exocytosis, propeptides are removed by specific proteolytic enzymes and tropocollagen is formed. Multiple tropocollagen molecules spontaneously assemble to larger collagen aggregates such as fibrils via covalent cross-links between hydroxylysine and lysine. Further, fibrilar bundles aggregate to collagen fibers. The tensile strength of fibrils is dependent on the cross-linking degree and varies from tissue to tissue. So far, 28 types of collagen have been identified and described at which collagen I is with over 90 % the most common type.

Fibronectin is a glycoprotein of the ECM consisting of two subunits, each of approximately 250 kDa linked by a pair of disulfide bonds. Despite being transcribed from the same gene, the monomers are not identical due to alternative splicing of its pre-mRNA leading to different isoforms. The monomers itself exhibit multiple functional and protein-binding domains each with specific binding sites for other matrix macromolecules as well as for the cell surface receptors called integrins. The existence of these domains in the fibronectin molecule affects the organization of the matrix as well as the cell attachment. The small tripeptide sequence Arg-Gly-Asp or RGD binds to the cell receptors integrin and mediates cell binding. Even the synthetically produced, short peptide containing the RGD sequence can compete with fibronectin for the cell binding site and can be coupled to solid surfaces. As an extracellular matrix protein fibronectin is secreted from the cells in soluble form initially. At the cell surface fibronectin monomers bind to integrins resulting in a stretching of the fibronectin molecule. With a partially unfolding of the protein and an unmasking of cryptic fibronectin binding-sites other fibronectin molecules can easily associate and form the insoluble fibronectin matrix.

Laminin is a glycoprotein and predominately found in the basal lamina, one of the layers of the basement membranes. These are thin sheets of extracellular matrix

that underlie epithelial and endothelial cells and surround muscle cells, Schwann cells and fat cells. The cross- or T-shaped heterotrimers of laminin consist of one α, one ß and one γ chain which are held together by disulfide bonds. So far, five α, three ß and three γ chains have been identified in vertebrates. On the globular domains of the three shorter arms of the peptide other laminin molecules can bind and assemble to sheets independently and are associated with type IV collagen networks. The longer arm containing the intersected ß and γ chain helps to anchor cells through the integrin receptors to the membrane. The laminin heterotrimers are assembled inside the cell, secreted and processed by proteolytic enzymes before reaching their final form.

Heparin belongs to the glycosaminoglycans, which are unbranched polysaccharids composed of repeated disaccharide units. More precisely, the repeating disaccharide units consist of glucoronic or iduronic acid linked to N-acetylglucosamine or N-acetylgalactosamine, which in most cases is sulfated. Because of the highly sulfation of most of their disaccharides, heparin has the highest negative charge density of any known biological molecule [102]. Therefore, heparin is important for the coupling of growth factors [103, 104].

For three-dimensional cell migration during physiologic and pathophysiologic processes, such as morphogenesis and regeneration as well as tumor invasion and metastasis cells developed strategies to pass the ECM. Therefore, the secretion of enzymes with proteolytic activity degrading the ECM is one possible option. Among others, matrix metalloproteinases (MMPs) are a widely studied calcium-dependent and zinc-containing family of endopeptidases participating in ECM degradation. To date, 24 different MMPs have been identified in vertebrates [105, 106]. They are secreted as inactive pro-MMPs and require an activation process before they developed their proteolytic activity [107]. Tissue inhibitors of matrix metalloproteinases (TIMPs) are also secreted proteins regulating the activity of MMPs [108]. The balance between MMPs and TIMPs regulates ECM degradation [109]. MMPs are expressed during development and adulthood in the CNS and are known for promoting CNS disease [110].

1.3.2 Occurrence in the central nervous system

For a long time, the existence of the ECM in the brain had been discussed controversially. It is now clear, that the extracellular space accounting for nearly 20 % of the adult brain is composed of many types of extracellular matrix molecules. In the central nervous system the ECM is responsible for the regulation of several processes both during development and throughout adulthood. Collagen as the major component of the ECM in the periphery is only rarely prevalent in the mature nervous system. Marginally it is found in the connective tissue associated with the nervous system, the basement membrane between the nervous system, and other tissues and the sensory end organs [111]. In humans collagen I and collagen II is present in the meninges, which is a system of membranes protecting the CNS [112, 113]. The glycoprotein fibronectin is associated with active morphogenesis, cell migration and inflammation, and is expressed in a diverse set of cells *in vitro* [114, 115]. It is also found in the developing cortex, especially in neurons migrating into specific cortical domains and is thought to support cell division and determine cell fate [116]. Punctated laminin expression in various regions has been described suggesting a possible role in axon guidance and neuron migration [117]. Beside an expression of laminin in the ependymal layer of the spinal cord it is also found along the growing fiber tracts [118-120]. During brain development and throughout adulthood laminin is expressed in different patterns, such as somatic or sheath form as well as small or large punctiform, each in conjunction with different stages of neuronal development [121].

1.3.3 Role of ECM in cellular processes

Cell growth, differentiation and migration are affected by signals from the ECM in vitro and in vivo [122-124]. Disrupted cell-matrix interactions are critical for the development of the CNS and can cause severe developmental defects [122]. ECM influences the proliferation and differentiation of murine neuroepithelial cells and neural stem and progenitor cells *in vitro* [125, 126] as well as the neuronal differentiation of postnatal neural stem/progenitor cells in a three-dimensional collagen-hyaluronan matrix [127]. The migration of mouse cerebellar neural precursor cells in vitro is also affected by ECM molecule [123]. Recently, the effects of several ECM components on the survival, proliferation, migration and process extensions of

rat oligodendrocyte progenitor cells have been reported [128]. Furthermore, laminin as a key ECM molecule enhance neural progenitor generation, expansion and differentiation into neurons from human embryonic stem cells [129].

1.4 Tissue engineering strategies for Parkinson's disease

1.4.1 Properties of an ideal biomaterial scaffold promoting protection, repair and regeneration in the brain

In the last years biomaterials were developed to restore the structure and function of damaged or dysfunctional tissue both in cell-based and in non-cellular therapies and play central roles in modern strategies in regenerative medicine and tissue engineering. In tissue engineering a principle task for biomaterial scaffolds is the regulation of cell behavior and tissue progression. For an application in Parkinson's disease several issues has to be considered in the design of an ideal biomaterial scaffold (Figure 2) [130].

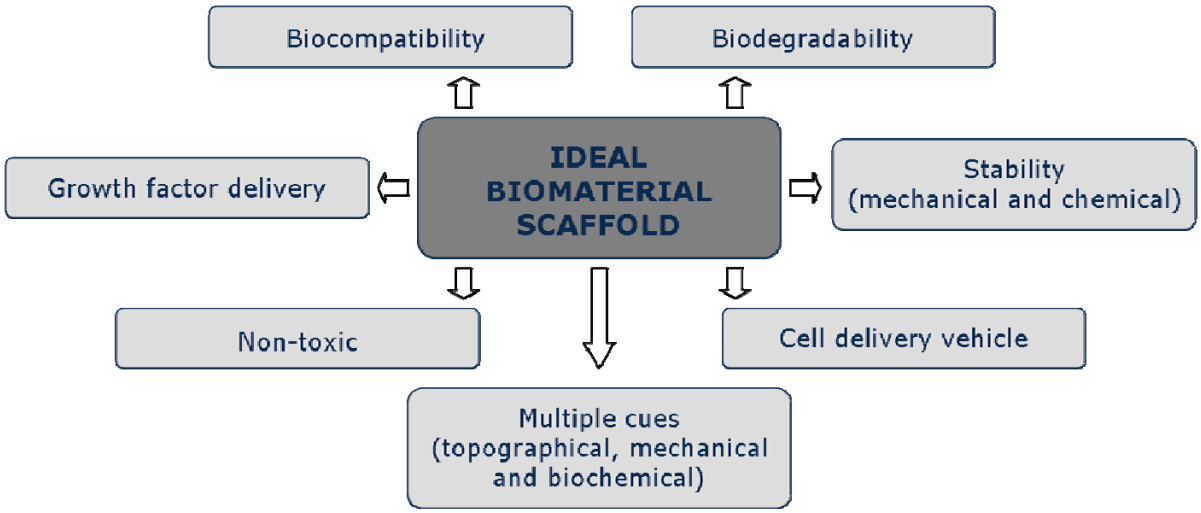

Figure 2: Properties of an ideal biomaterial scaffold for applications in Parkinson's disease. An ideal biomaterial scaffold has to fulfill several requirements to restore the structure and the function of the damaged tissue in the brain (modified from [130]).

These materials should be non-toxic to the brain tissue and chemically and mechanically stable long enough to perform its biological function by supporting structurally the surrounding tissue and promoting local neurite and axonal outgrowth.

After successful repair or regeneration of the tissue the material should ideally degrade without any toxic residues remaining. To prevent inflammatory response or cellular reaction biomaterials should be biocompatible with low immunogenicity. Moreover, for cell replacement of the lost cell type in PD the biomaterial should serve as cell delivery vehicle with various requirements. Ideally it provides controllable release of growth factors or bioactive compounds spatially and temporally at the side of implantation. Finally, it should serve as three-dimensional extracellular microenvironment mimicking all the topographical, mechanical and biochemical cues of natural extracellular matrix (EMC). Thus, the biomaterials can promote cell adhesion, viability and proliferation as well as provide axon guidance cues for neurite extension.

1.4.2 Mimicry of the extracellular matrix as guideline for bioscaffold design

While the currently applied scaffold materials are mostly based on one main structural component [131-134], natural ECM consists of a variety of complex supramolecular assemblies of various proteins, proteoglycans and glycosaminoglycans [135]. Although the ECM serves as storage system for several morphogens in most of the currently applied bioscaffols these molecules are still missing. To support effectively cellular multistep processes, engineered matrices have to mimic these characteristics more closely. Recently, more advanced materials were designed comprising multiple sets of ECM components [136-139] and releasing signaling molecules sequentially [140, 141]. Besides combining biomolecules of the ECM with scaffold materials enabling cell attachment and morphogen release, the strong impact of mechanical properties of ECM on cellular processes were shown recently [142]. Accordingly, the defined and systematic variation of the biomolecular complexity and the mechanical characteristics of biofunctional matrices are expected to open new options for cell replacement therapies. Moreover, engineered biodegradable scaffolds could be used to promote and guide axonal outgrowth from the transplanted tissue within the mesencephalon into the striatum and thus reconstruct the nigro-striatal pathway.

1.4.3 starPEG-heparin hydrogels as cell-based and drug delivery system

Hydrogels received much attention due to their use in a variety of tissue engineering applications. They are characterized by a three-dimensional network of hydrophilic polymers and thus are able to retain huge amounts of water without destructing their polymer network. In the swollen state of hydrogels the polymer content is typically below 10 % of weight, whereas 90 % of the remaining mass is water. This high water content is similar to natural soft tissue and, hence, exhibit enhanced biocompatibility. Moreover, it facilitates the exchange of oxygen, nutrients and waste which is often critical for supporting long-term cell/tissue viability in tissue regeneration. On the other hand, the high water content in hydrogels may not prevent cytotoxic molecules, including cytokines and reactive oxygen species, to penetrate into the hydrogel and triggering apoptotic pathways.

A wide range of synthetic and naturally derived materials forming hydrogels has been developed for the use in tissue engineering strategies. Naturally derived polymers including agarose, alginate, chitosan, collagen, fibrin, gelatin and hyaluronic acid have frequently been used because of similarly macromolecular properties to natural ECM [131]. They can provide abundant biological signals, such as for cell adhesion, and degrade into physiological harmless compounds. On the other hand, the can provoke inflammatory and immunological reactions. The combination of naturally derived materials with polysaccharide molecules, such as hyaluronic acid and heparin has been widely used [143-147]. The glycosaminoglycan heparin with its high anionic character is used due to its affinity to many signaling molecules. Binding of growth factors such as FGF-2 and GDNF to heparin mainly occurs though electrostatic interactions between the negatively charged *N*- and *O*-sulfated groups of heparin to lysine and arginine residues of FGF-2 and GDNF [148, 149]. Growth factors are characterized by a rapid degradation upon direct administration. Binding of FGF-2 and GDNF to heparin leads to a protection of their bioactivities against proteolytic modifications [149, 150].

Besides natural polymers, synthetic hydrogels exhibit great potential for cell replacement therapies due to their controllable chemistry and properties enabling a defined and systematic variation of mechanical characteristics and biomolecular functionalization [151]. Furthermore, synthetic hydrogels minimize the risk for

immune response. Among other synthetic polymers, poly (ethylene glycol) (PEG) based hydrogels have been extensively used as cell and drug delivery vehicles for promoting tissue regeneration [131, 152, 153] since they offer a high biocompatibility [154]. Moreover, the polymer is characterized by a hydrophilic and uncharged nature and the possibility to easily modify its terminal end groups [133]. Recently, a biohybrid hydrogel based on covalently cross-linked heparin and star-shaped poly(ethylene glycol) (starPEG) has been developed [155]. Three-dimensional networks were formed by cross-linking of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysulfo-succinimid (EDC/s-NHS)-activated carboxylic acid groups of heparin with amino end-functionalized starPEG. Network characteristics and biomolecular functionalization can be gradually and independently tuned. Cross-linked heparin molecules within the three-dimensional system were subsequently functionalized through covalent attachment of the cell adhesion mediating peptide RGD and non-covalent binding of growth factors (Figure 3). The implantation of the biohybrid hydrogel in the brain proved its feasibility, stability and biocompatibility [155].

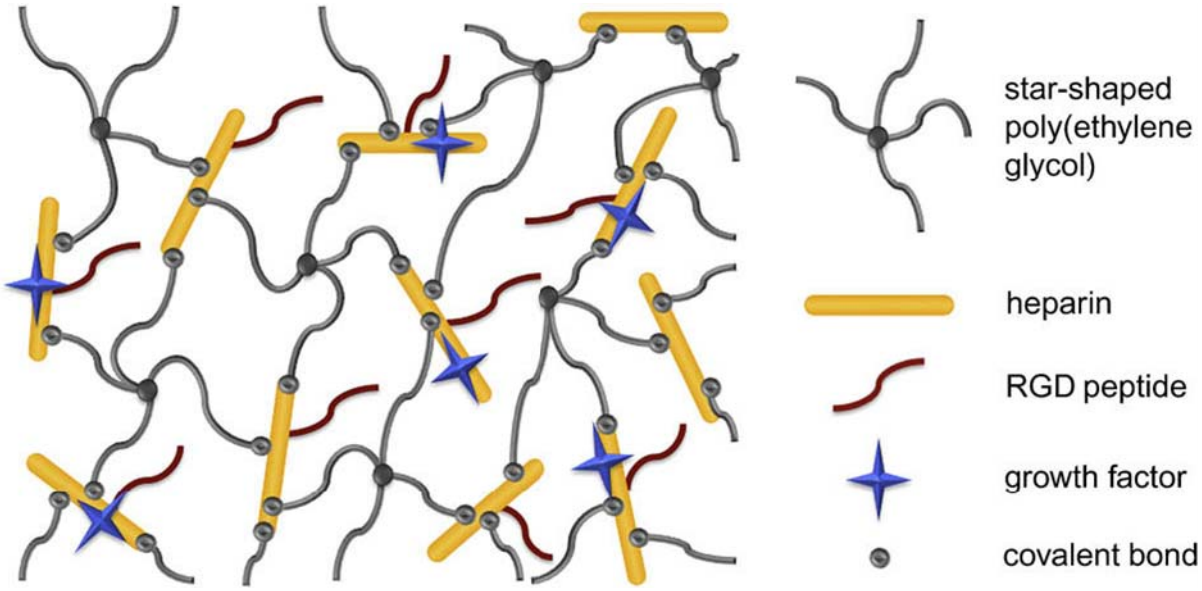

Figure 3: Design of EDC/s-NHS activated heparin cross-linked with amino end-functionalized starPEG to form a non-cleavable biohybrid hydrogel. Additionally, the three-dimensional network system can be functionalized with the cell adhesion ligand RGD and soluble signaling molecules like growth factors [155].

Furthermore, starPEG-heparin hydrogels were shown to be an efficient FGF-2 storage and delivery system with adjustable release characteristics decoupled from the network structure [156]. Furthermore, for this hydrogel system the independent delivery of two growth factors simultaneously has been demonstrated [157]. This affinity-based heparin binding delivery system utilizes a non-covalent interaction of growth factors with the scaffold providing a controlled protein release. Thus, any protein that binds to heparin can be delivered. Many other methods of drug delivery have been developed for nerve tissue engineering [158].

As three-dimensional cell migration through the ECM needs cell adhesion [159] as well as proteolytic degradation [160] events, these molecular signals has to be displayed by synthetic materials. Matrix metalloproteiases (MMPs) has been identified to play a key role in cell invasion and tissue remodeling [161, 162]. So far, 24 different MMPs have been identified in vertebrates, of which 23 exist in humans [106]. They are secreted as pro-MMP (zymogen) and needs to be activated before they are able to cleave the ECM [107]. Tissue inhibitors of metalloproteinase (TIMP) regulate the MMP activity, and thus the balance between both proteins controls the degradation of the ECM [108, 163].

Oligopeptide substances for MMPs were cross-linked with multiarm end-functionalized PEG macromers to a three dimensional network [164]. Proteolytic cell invasion into these networks has been demonstrated by primary human fibroblasts [165]. Recently, a modular biohybrid hydrogel could be formed consisting of heparin and peptide-conjugated starPEG cross-linked by MMP sensitive peptides [166] (Figure 4). Biomodifications of enzymatically degradable starPEG-heparin hydrogels with the cell adhesion mediating ligand RGD and a growth factor support the three-dimensional migration of human endothelial cells [167]. The protease sensitivity in the PEG hydrogels makes this system interesting for an application in PD.

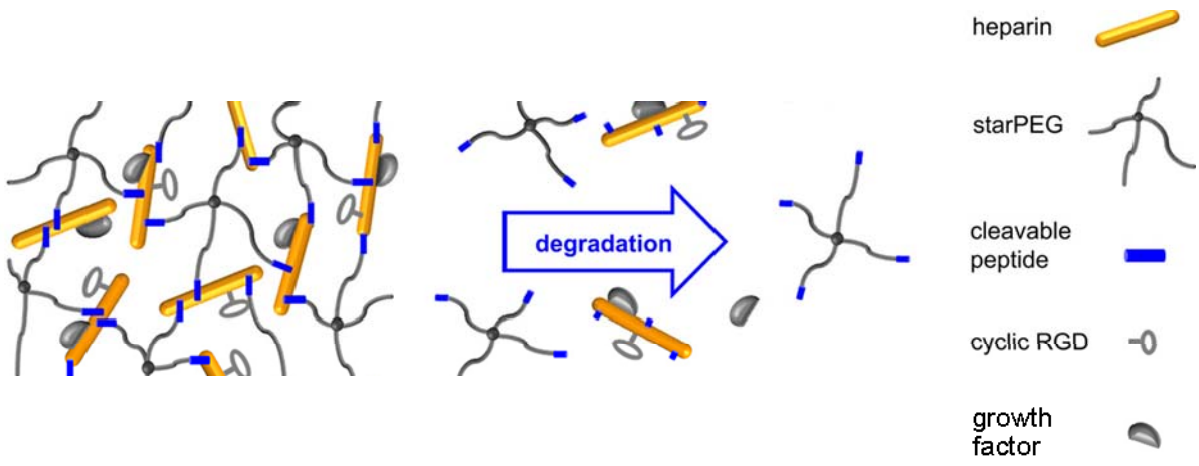

Figure 4: Design of EDC/s-NHS activated heparin cross-linked with amino end-functionalized peptide-conjugated starPEG to form a cleavable biohybrid hydrogel. Additionally, the three-dimensional network system can be functionalized with the cell adhesion ligand RGD and soluble signaling molecules like growth factors (modified by [168]).

1.5 Aim of the study

Neurotransplantation in PD is often hampered by a low cell survival and the lacking integration of the grafted cells into the nigro-striatal neuronal circuitries due to the *ectopic* transplantation of cells into the striatum. To overcome these problems the transplantation of cells *orthotopically* into their natural position within the *Substantia nigra* could be an alternative approach in PD. For reconstructing the central dopaminergic nigro-striatal pathway a biomaterial could be implanted as artificial nerve pathway in a so called “bridging” technology to promote dopaminergic outgrowth from the *Substantia nigra* and to re-innervate the striatum. The aim of the thesis was to provide the scientific basis for the use of injectable bioscaffolds containing chemo attractants modulating cell survival, differentiation and axo-dendritic outgrowth of dopaminergic cells. Bridging the distance between *Substantia nigra* and striatum with a biomaterial promoting axo-dendritic outgrowth of grafted dopaminergic neurons would provide a new regeneration approach in PD.

Hence, the first step of this work was to elucidate the influence of several ECM substrates and the cell adhesion mediating peptide RGD on the axo-dendritic outgrowth of dopaminergic cells derived from primary fetal mesencephalic cells. For a more precise analysis of the dopaminergic cell morphology three different cell

characteristics have been investigated including primary branching, total branching and neurite elongation. The second element addressed the interaction of primary fetal mesencephalic cells and fetal mesencephalic NSCs with the newly developed starPEG-heparin hydrogel. The gel system can be modulated in its chemical/physical and biomolecular composition and thus is adjustable to a wide range of applications. The effects of several hydrogel settings including various material stiffness and biomolecular functionalization on the survival of both cellular sources were explored. Later, main focus concentrated on primary fetal mesencephalic cells and their interaction with hydrogels applicable for an injection into the brain. This included cell culture studies with soft hydrogels and their additional modifications with a growth factor relevant in terms of PD. Thereby, survival studies, especially of various cell types were relevant.

Within the third part of this work the ability of hydrogels serving as an efficient storage and delivery system for growth factors were investigated. Analyses consist of the separate as well as simultaneous immobilization and release of two distinct growth factors. The last part of the work addressed the question whether primary fetal mesencephalic cells show the ability to degrade starPEG-heparin hydrogels containing proteolytically cleavable peptide linkers. Thereby, the expression of MMPs, ECM degrading proteinases was analyzed. Penetration of cells into the cleavable hydrogel was determined in comparison to non-cleavable hydrogel to proof their biodegradability by primary midbrain cells.

The results of this work could be beneficial to improve cell survival after neural transplantation and to facilitate three-dimensional cell growth with the use of a cleavable hydrogel being an ideal bioscaffold for an artificial nerve pathway. This could be a promising approach for the treatment of PD using the “bridging” transplantation technology.

2. Material and Methods

2.1 Material

2.1.1 Instruments

Instrument	Designation	Company
Balance	SBA 52	Scaltec (Göttingen, Germany)
Cell incubator	Hera Cell 150	Heidolph (Düsseldorf, Germany)
Cell Prep Instruments	various	VWR (Darmstadt, Germany)
Centrifuge, small	Mini Spin Plus	Eppendorf (Hamburg, Germany)
Centrifuge, middle	Biofuge	Heraeus (Berlin, Germany)
Clean bench	Hera Safe	Heraeus (Berlin, Germany)
Confocal microscope	SP5	Leica Microsystems (Braunschweig, Germany)
Dissection microscope		Zeiss (Oberkochen, Germany)
Fluorescence microscope	DM IRE2	Leica Microsystems (Braunschweig, Germany)
Immobilization chamber		customer-made
PCR cycler	MX 3000 P	Stratagene (La Jolla, USA)
Spin coater module	RC5	Suess Microtec (Garching, Germany)
Thermomixer	Comfort	Eppendorf (Hamburg, Germany)
Vortexer	L 46	GLW (Würzburg, Germany)
Water bath	SW 22	Julabo (Seelbach, Germany)

2.1.2 Chemicals and Consumables

Substance or Consumable	Company
3-Aminopropyltriethoxysilane	Sigma-Aldrich (St.Louis, USA)
Ammonium hydroxide	Acros Organics (Geel, Belgium)
BSA	Serva (Heidelberg)
Collagen I	Vitrogen (Palo Alto, USA)
Cyclo(Arg-Gly-Asp-D-Tyr-Lys) (cyclic RGD)	Peptides International (Louisville, USA)

1-Ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrobromide (EDC)	Sigma-Aldrich (St. Louis, USA)
Ethanol	Roth (Karlsruhe)
Fibronectin	Roche (Basel, Switzerland)
Glucose	Sigma-Aldrich (St. Louis, USA)
Heparin (for collagen/heparin)	Sigma-Aldrich (St. Louis, USA)
Heparin (MW 14,000 – for starPEG)	Merck (Darmstadt, Germany)
Hydrochloric acid	Merck (Darmstadt, Germany)
Hydrogen peroxide	Merck (Darmstadt, Germany)
Immuno 96 MicroWell™ Solid Plates (MaxiSorp™)	Thermo Fisher Scientific (Rochester, USA)
Isopropanol	Merck (Darmstadt, Germany)
Laminin	Roche (Basel, Switzerland)
2-mercaptoethanol	Sigma-Aldrich (St. Louis, USA)
N-hydroxysulfosuccinimide (s-NHS)	Sigma-Aldrich (St. Louis, USA)
Paraformaldehyde (PFA)	Sigma-Aldrich (St. Louis, USA)
Poly-D-lysine	Sigma-Aldrich (St. Louis, USA)
Sodium bicarbonate	Sigma-Aldrich (St. Louis, USA)
Sodium carbonate	Merck (Darmstadt, Germany)
Poly(ethylene glycol) (starPEG) (MW 10,000)	Polymer Source (Dorva, Canada)
Poly(ethylene glycol) (starPEG) (MW 15,200)	Polymer Source (Dorva, Canada)
Poly(octadecene-alt-maleic anhydride) (POMA)	Polysciences Inc. (Warrington, USA)
Triton-X 100	Thermo Fisher Scientific (Rockford, USA)

2.1.3 Solutions and Buffers

Substance or consumable	Company
Accustain	Sigma-Aldrich (St. Louis, USA)
Donkey serum	Jackson Immunoresearch (West Grove, USA)
HBSS	Gibco (Invitrogen, Carlsbad, USA)
Hoechst 33342	Molecular Probes (Invitrogen, Carlsbad,

	USA)
Hepes buffer	Sigma-Aldrich (St. Louis, USA)
PBS	Gibco (Invitrogen, Carlsbad, USA)
Propidium iodide (PI)	Molecular Probes (Invitrogen, Carlsbad, USA)
RNAlater®	Ambion® (Austin, USA)
Sigmacote	Sigma-Aldrich (St. Louis, USA)
TBST wash buffer	Dako (Glostrup, Denmark)
Ultra Pure (PCR-) Water	Roth (Karlsruhe, Germany)
Vectashield	Vector (Burlingame, USA)

2.1.4 Kits

Kit	Company
Brilliant ® SYBR® Green QRT-PCR Master Mix	Stratagene (La Jolla, USA)
RNeasy ® Mini	Qiagen (Hilden, Germany)
RNeasy ® Lipid Tissue Mini Kit	Qiagen (Hilden, Germany)
Human FGF basic Quantikine ELISA Kit	R&D Systems (Minneapolis, USA)
GDNF E_{max}® Immuno Assay System	Promega (Madison, USA)

2.1.5 Enzymes, Growth factors and Antibodies

Enzymes

Enzymes	Company
DNase (RNA isolation)	Qiagen (Hilden, Germany)
DNase (cell preparation – fetal primary mesencephalic cells)	Roche (Grenzach-Wyhlen, Germany)
DNase (cell preparation – fetal mesencephalic neural stem cells)	Sigma-Aldrich (St. Louis, USA)
Trypsin	Gibco (Invitrogen, Carlsbad, USA)

Growth factors

Growth factors	Company
EGF (human, recombinant)	Sigma-Aldrich (St. Louis, USA)
GDNF (human, recombinant)	Sigma-Aldrich (St. Louis, USA)
FGF-2 (human, recombinant)	Sigma-Aldrich (St. Louis, USA)
Forskolin (human, recombinant)	Sigma-Aldrich (St. Louis, USA)
IL-1b (Interleukin-1) (human, recombinant)	Sigma-Aldrich (St. Louis, USA)

Antibodies

Primary Antibodies

Antibody	Dilution	Company
Mouse anti-microtubuli-associated protein 2 (MAP2)	1:500	BD Biosciences (Franklin, USA)
Mouse anti-ß-Tubulin III (Tuj1)	1:500	Millipore (Billerica, USA)
Rabbit anti-tyrosine hydroxylase (TH)	1:500	PelFreeze (Rogers, USA)
Rabbit anti-Nestin	1:500	Abcam (Cambridge, UK)
Rabbit anti-Olig2	1:500	Chemicon (Temecula, Canada)
Rabbit anti-Glial Fibrillary Acidic Protein (GFAP)	1:1000	Millipore (Billerica, USA)
Rabbit anti-matrix metalloproteinase-2 (MMP-2)	1:100	Abcam (Cambridge, UK)
Rabbit anti- matrix metalloproteinase-9 (MMP-9)	1:100	Abcam (Cambridge, UK)
Sheep anti-tyrosine hydroxylase (TH)	1:500	PelFreeze (Rogers, USA)

Secondary Antibodies

Antibody		Company
Alexa Fluor® 488-conjugated donkey anti-mouse	1:500	Invitrogen (Carlsbad, USA)
Alexa Fluor® 488-conjugated donkey anti-sheep	1:500	Invitrogen (Carlsbad, USA)
Alexa Fluor® 594-conjugated donkey anti-mouse	1:500	Invitrogen (Carlsbad, USA)
Alexa Fluor® 594-conjugated donkey anti-rabbit	1:500	Invitrogen (Carlsbad, USA)

CF633 phalloidin conjugate	1:40	Biotium (Hayward, USA)

2.1.6 qRT-PCR Primers

Primer	Quiagen (Hilden, Germany) (not specified by manufacturer)
MMP-2	QuantiTect®Primer Assay Mm_MMP2_1_SG
MMP-9	QuantiTect®Primer Assay Mm_MMP9_1_SG

2.1.7 Cell Culture Media

Expansion medium for fetal mesencephalic neural stem cells (NSCs)

Medium component	Company
65% (v/v) DMEM	Gibco (Tulsa, UK)
((+)4.5g/l Glucose, (+) L-Glutamine, (+) Pyruvat)	
32% (v/v) F-12+GlutaMAX™ ((-) L-Glutamine)	Gibco (Tulsa, UK)
2 % B-27 Supplement	Gibco (Tulsa, UK)
100 IU/ml Penicillin	Gibco (Tulsa, UK)
0.1 mg/ml Streptomycin	Gibco (Tulsa, UK)
20 ng/µl EGF	Sigma-Aldrich (St. Louis, USA)
20 ng/µl FGF-2	Sigma-Aldrich (St. Louis, USA)

Differentiation media for fetal mesencephalic neural stem cels (NSCs)

Medium component	Company
65% (v/v) DMEM	Gibco (Tulsa, UK)
((+)4.5g/l Glucose, (+) L-Glutamine, (+) Pyruvat)	
32% (v/v) F-12+GlutaMAX™	Gibco (Tulsa, UK)
((-) L-Glutamine)	
2 % B-27 Supplement	Gibco (Tulsa, UK)
100 IU/ml Penicillin	Gibco (Tulsa, UK)
0.1 mg/ml Streptomycin	Gibco (Tulsa, UK)
5 µM Forskolin (human, recombinant)	Sigma-Aldrich (St. Louis, USA)
100 pg/ml IL-1b (Interleukin-1) (human, recombinant)	Sigma-Aldrich (St. Louis, USA)

Basic-Medium for primary fetal mesencephalic cells

Medium component	Company
87% (v/v) DMEM	Gibco (Tulsa, UK)
((+)4.5g/l Glucose, (+) L-Glutamine, (-) Pyruvat)	
10% (v/v) FCS	Sigma-Aldrich (St. Louis, USA)
10 mM Hepes buffer	Gibco (Tulsa, UK)
8 mM Glucose	Sigma-Aldrich (St. Louis, USA)
100 IU/ml Penicillin	Gibco (Tulsa, UK)
0.1 mg/ml Streptomycin	Gibco (Tulsa, UK)

2.1.8 Animals

Male and female wild-type C57Bl6 (25-35 g) were purchased from Charles River, Sulzfeld. They were housed, bred and treated according to the guidelines of the European Community (86/609/EEC) and the Society for Neuroscience (January 1985). All experiments were approved by the local ethical committee. Timed pregnant females were obtained by overnight mating. The day of detection of the vaginal plug was considered as E1.

2.2 Methods

2.2.1 Preparation of hydrophobic glass coverslips

The surface of the glass cover slips to be siliconized must be clean and dry. Cover glasses in a Petridish with enough Sigmacote solution for 15 sec and remove the solution afterwards. The silicone solution forms a covalent thin film on the glass surface which is water repellent. Allow the treated glass surface to air dry in a hood. After the heptane has evaporated, the siliconized glasses can be oven dried at 100 °C for 30 min. Rinse the siliconized glass cover slips with MilliQ water to remove the HCl by-products before use.

2.2.2 Preparation of aminosilanized glass cover slips

1) Pre-cleaning

To remove particles from the glass surface cover slips were immersed in MilliQ water in a Teflon shelf, incubated for 30 min in MilliQ water in an ultrasonic water bath and rinsed 2-3x in MilliQ water. To further pre-clean the glass cover slips were incubated in Ethanol in an ultrasonic water bath and rinsed 2-3x in MilliQ water subsequently.

2) RCA-Cleaning

Cover slips were immersed in a Teflon shelf with pre-heated (70 °C) RCA solution (volume ratio of MilliQ water : H_2O_2 : NH_3 = 5 : 1 : 1) for 10 min and rinsed 2-3x in MilliQ water subsequently. After cleaning the glass cover slips should be further processed immediately because fast re-contamination processes occurring at environmental conditions.

3) Aminosilanization

Cover slips were placed in isopropanol : MilliQ water (9 : 1) solution containing 20mM 3-aminopropyltriethoxysilane in a Teflon shelf for 2 h. After rinsing samples in isopropanol thoroughly the glass surface were dried with N_2 gas and placed in a drying oven at 120 °C for 1 h. Because of attacking the amino groups by oxidation the drying step and subsequent gel formation should be carried out in a shortly after aminosilanization.

2.2.3 Covalent protein immobilization of ECM components and poly-D-lysine on surface modified glass cover slips

In a first step a thin film of 0.16 % poly(octadecen-*alt*-maleic anhydride) (POMA) solution in THF were spin coated at 4000 rpm for 30 s on the surface of freshly prepared aminolisanized glass cover slips by using a spin coater module. For a stable covalent binding of polymers to the SiO_2 surface of glass cover slips samples were placed at 120 °C for 2 h in an oven, where imide bonds with the aminosilane of the pre-coated cover slips were generated. Later, glass cover slips were flushed in acetone for 15 min and were dried with N_2 gas immediately. Before proteins of the

ECM were covalently immobilized to the modified glass surface, cover slips were autoclaved at 120 °C for 20 min and subsequently oven dried at 120 °C for 2 h.

Collagen fibrils were reconstituted according to the manufacturer's manual and diluted further as follows: collagen I : 10 x PBS : 0.1 M NaOH = 8 : 1 : 1. Components were mixed and kept on ice. By adding ice cold PBS the appropriate concentration was adjusted. The collagen solution was placed on the modified glass surface and formation of fibrils was initiated by incubation for 1 h at 37 °C.

For tropocollagen I coating acidic collagen I solution were incubated on modified glass surface overnight.

For preparation of collagen I / heparin coatings non ice cold fibrillar collagen I solution were mixed with in PBS diluted heparin. The mixture were incubated for 15 min on ice and placed on modified glass surfaces. For the formation of fibrils the cover slips were incubated for 1 h at 37 °C.

Laminin were diluted according to the manufacturer's manual and concentration were adjusted by adding an appropriate volume of DMEM ((+)4.5g/l Glucose, (+) L-Glutamine, (-) Pyruvat). After placing the laminin solution on glass surface the cover slips were incubated for 1 h at 37 °C.

In PBS diluted fibronectin were placed on modified glass surface and incubated for 1 h at room temperature.

RGD dissolved in borate buffer (pH 8) were placed on modified glass surface and incubated for 2 h at room temperature.

After appropriate incubation the cover slips were washed 3 times with PBS. Following concentrations were used: collagen I (1.2 mg/ml), tropocollagen I (0.1 mg/ml), collagen I / heparin (1.2/0.4 mg/ml), laminin (0.01 mg/ml), fibronectin (0.05 mg/ml), RGD (0.2 mg/ml) and poly-D-lysine (0.05 mg/ml).

2.2.4 Preparation of functionalized starPEG-heparin hydrogels

For covalent binding of starPEG with heparin an activation of heparin carboxylic acid groups by EDC and s-NHS is necessary prior combining both matrix components. To obtain surface immobilized networks the starPEG-heparin hydrogel solution were placed on freshly amino end-functionalized glass cover slips. The activated carboxylic acid groups of heparin attach covalently and anchor the hydrogel to the modified glass surface. In order to spread the gel solution equally on the glass slide

the gel solution was covered with a hydrophobic glass cover slip. After polymerization of the hydrogel the hydrophobic cover slip was removed.

Preparation of non-cleavable starPEG-heparin hydrogel networks

By cross-linking amino end-functionalized four-arm starPEG with EDC/s-NHS-activated carboxylic acid groups of heparin the starPEG-heparin hydrogels were formed. For this total polymer content of 11.6% and a molar ratio of EDC and s-NHS of 2 : 1 were used. To generate gels with different physical and chemical characteristics the molar ratio of starPEG (MW 10,000) to heparin (γ=2, γ=4, γ=6) was varied. First, starPEG and heparin were each dissolved in one third of the total volume of ice-cold MilliQ water by ultrasonication and stored on ice. After EDC and s-NHS were dissolved in one sixth of the total volume in ice-cold MilliQ water the solution were added to heparin, mixed and incubated for 15 min on ice. After heparin carboxylic acid groups were activated the starPEG solution were added and mixed for 15 min at 8 °C and 900 rpm. Subsequently, 3.11 µl of the gel mixture were placed on aminosilanized glass cover slips to allow covalent attachment of heparin through its activated carboxylic acid groups. In order to spread the gel solution equally on the glass slide the gel solution was covered with a hydrophobic glass cover slip. After polymerization of the hydrogel over night at 22 °C the hydrophobic cover slip was removed and hydrogels were washed five times with PBS to remove any remaining, non-bound matrix components. After intensive washing the gels were stored for 24 h in PBS to initialize gel swelling. For cell culture the swollen gels were sterilized using UV-treatment for 30 min.

Biomodification of non-cleavable starPEG-heparin hydrogels

PBS-swollen gels were washed three times for 10 min in 1/15 mM phosphate buffer (pH = 5) at 4 °C. For activation of the carboxylic acid groups of heparin and a subsequent biomodification with cyclo (Arg-Gly-Asp-D-Tyr-Lys) (RGD) a solution of 50 mM EDC and 25 mM s-NHS in 1/15 mM phosphate buffer were added and gels were incubated for 45 min at 4 °C. Afterwards, the gels were washed 3 times in 100 mM borate buffer (pH = 8, 4 °C) to remove excessive EDC/s-NHS and immediately immersed in 250 µl RGD-solution (50µg/ml in 100 mM borate buffer) to finally reach an amount of 15 µg RGD bound to the network. After incubation for 2h at room

temperature the samples were washed 3 times in PBS for 1 min. For immobilization of growth factors (FGF-2 and/or GDNF) to the heparin hydrogel the particular protein were dissolved in PBS at the desired concentration of 1 µg/ml. Swollen pure or RGD modified gels were immersed in 200µl/cm^2 FGF-2 and/or GDNF solution at room temperature for 6 h followed by a washing in PBS twice.

Preparation of cleavable starPEG-heparin hydrogel networks

The generation of biodegradable gels was performed as described for the non-cleavable gels using starPEG-MMP conjugates with a molecular weight of PEG of 15,200 Da. Especially, amide conjugated MMP-cleavable peptides with the cleavable sequence GPQG↑↓IWGQ were used. Biodegradable gels were functionalized with cyclic RGD with a final amount of 4 or 32 µg.

2.2.5 Isolation and cultivation of primary fetal mesencephalic cells

Time-pregnant females at embryonic day 14 (E14) were killed according to National Institutes of Health guidelines and with approval of the local animal care committee. Primary fetal cells were isolated from the mesencephalic tegmentum using a dissection microscope. After removing the meninges carefully the tissue samples were cut with a scalpel into small pieces and incubated with 2 ml 0.5% Trypsin/EDTA, 3ml HBSS and 200 µg/ml DNAse for 7min at 37 °C. Samples were centrifuged for 2 min at 500 x g and the supernatant was removed. For dissociation of cells 3 ml pre-warmed Basic medium and 200 µg/ml DNAse were added and samples were homogenized to a quasi single cell suspension by mechanical trituration using a fire polished Pasteur pipette. After 10 min of incubation the supernatant containing the dissociated cells were removed. Existing cell pellets were re-suspended with 3 ml pre-warmed Basic medium and 200 µg/ml DNAse and cells were dissociated by mechanical trituration up to three times. Dissociated cells were seeded with a density of 2,500 cells/mm^2 to pre-coated cover slips or starPEG-heparin hydrogels. The complete medium was changed on the 3rd day in vitro (DIV) and on the 5th DIV. Primary fetal mesencephalic cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air (21% O2).

All incubation steps were performed at room temperature if not mentioned else wise.

2.2.6 Isolation, expansion and differentiation of fetal mesencephalic neural stem cells

Mesencephalic NSCs were dissected from mice at E14 as described above. After removal of the meninges the tissue samples were cut into small pieces with a scalpel, incubated in 0.1 % trypsin for 5 min, triturated and centrifuged for 4 min at 500 x g. The supernatant was removed, 40 mg/ml DNase was added and the sample was homogenized to a quasi single cell suspension by mechanical trituration using a fire polished Pasteur pipette. After incubation for 10 min at 37 °C the cell suspension were centrifuged for 4 min at 500 x g, the supernatant was discarded and cells were re-suspended in 8 ml pre-warmed expansion medium containing 20 ng/ml EGF and FGF-2. NSCs were expanded as suspension cultures in so-called “neurospheres” in 25 cm^2 flasks for one week at 37 °C in a humidified atmosphere of lowered oxygen conditions 5% CO2, 92% N_2 and 3%O_2 and growth factors (20 ng/ml EGF and FGF-2) were added twice a week. After 2 – 3 days in culture fetal neurosphere formation was observed.

To induce differentiation cells were transferred into a falcon tube and centrifuged for 4 min at 500 x g to remove the supernatant. After that, they were washed once by resuspension in 5 ml PBS followed by another centrifugation step for 4 min at 500 x g before 8 ml pre-warmed differentiation media containing 5 mM forskolin and 100 pg/ml interleukin (IL)-1b were added. Cells were transferred to pre-coated cover slips or starPEG-heparin hydrogels.

All steps were performed at room temperature if not mentioned else wise.

2.2.7 Immuncytochemistry

After 7DIV cells were washed once with PBS and subsequently fixed for 30 sec with Accustain solution. After rinsing three times with PBS cells were blocked against unspecific antibody labeling using blocking solution (3% donkey serum in PBS containing 0,2% TritonX-100) for 1.5 h. Primary antibodies diluted in blocking solution were added to the cells and incubated overnight at 4 °C. Subsequently, cells were rinsed three times with PBS for 10 min and incubated with the respective fluorophore-labeled secondary antibodies for 1 h in the dark. Afterwards cells were rinsed four times with PBS for 10 min and cell nuclei were counterstained with Hoechst 33342

(7,5 ng/ml) for 3 min. After washing cells three times with PBS cover slips were removed from the culture dish and mounted on microscopy slides using Vectashield.

For immunostaing of MMPs cells were once washed with PBS after 7 DIV, fixed for 15 min with 4% PFA on ice and subsequently rinsed three times with PBS. After blocking with 1% BSA (in PBST - containing 0.025% Triton) for 30 min, primary antibodies were added and cells were incubated overnight at 4 °C. Subsequently, cells were rinsed three times with PBS and incubated with the respective fluorophore-labeled secondary antibodies for 1 h in the dark followed by washing with PBS four times for 10 min. Counterstaining of the cell nuclei were performed as described above.

For phalloidin staining cells were washed once with PBS after 1 DIV or 5 DIV, fixed for 15 min with 4% PFA on ice and rinsed three times with PBS. To permeabilize the cell membrane 0.5% TritonX-100 was added and cells were incubated for 10 min followed by three times washing step with PBS. To block non-specific binding 3% BSA were added. After 30 min cells were washed once with PBS, the 633 conjugated phalloidin antibody in 1 % BSA were added and cells were incubated for 1 h in the dark. Cells were washed three times with PBS and cell nuclei were stained like mentioned above.

All incubation steps were performed at room temperature if not mentioned else wise.

2.2.8 Cell survival studies

Cell survival was analyzed by propidium iodide (PI) nucleic acid staining. PI is a fluorescent molecule and intercalates to DNA. Once the dye is bound to nucleic acid, its fluorescence is enhanced 20- to 30-fold, the fluorescence excitation maximum is shifted to 535 nm and the fluorescence emission maximum to 617nm. While the membrane of viable cells is impermeable for PI, the dye can penetrate cell membranes of dying or dead cells. This method is commonly used for identifying dead cells.

After 7DIV the cell culture medium was removed, cells were washed once with PBS and a solution containing 325 µl PBS, 125 µl cell culture medium, 80 ng/ml PI

and 4 ng/ml Hoechst 33342 were added to each sample. Cells were incubated with the intercalating agents for 6 min at 37 °C and subsequently the fluorescence was measured.

2.2.9 Isolation of RNA

Isolation of RNA from adherent cells

Adherent cells were detached from their culture dish using a cell scraper after 7 DIV on different substrates. The cell solution was transferred in a falcon tube and centrifuged for 4 min at 316 x g. After removal of the supernatant the cell pellet was re-suspended with PBS. The cell solution was centrifuged once again for 1 min at 316 x g and the cell pellet were re-suspended in 350 µl RLT lysis buffer containing 1 % 2-mercaptoethanol. Isolation of RNA from cells was performed with the RNeasy® Mini Kit followed by elution with RNase-free DNase according to the manufacturer's manual. In this procedure, RNA is bound to a silica gel membrane and washed several times to remove contaminants and enzyme inhibitors before elution.

Isolation of RNA from tissue

Tissue from brain regions of interest were placed in 500 µl RNAlater™ solution. After transferring samples into 500µl of Quizol-lysis-buffer the tissue was homogenized. Isolation of RNA from cells was performed with the RNeasy Mini Kit followed by elution with RNase-free DNase according to the manufacturer's manual. In this procedure, RNA is bound to a silica gel membrane and washed several times to remove contaminants and enzyme inhibitors before elution.

2.2.10 Quantitative real-time reverse transcription PCR (qRT-PCR)

Quantitative real-time reverse transcription PCR (qRT-PCR) is a powerful tool for gene expression analyses. By the combination of real time PCR with reverse transcription messenger RNA and non-coding RNA in cells and tissue can be amplified and simultaneously quantified.

QRT-PCR was performed using Brilliant® SYBR® Green QRT-PCR Master Mix Kit according to the manufacturer's manual. The fluorophore SYBR® Green bind to all double-stranded DNA (dsDNA) molecules regardless of sequence and enhance

the fluorescence emission due to a conformational change in the dye. During denaturation, all DNA becomes single-stranded. At this stage, SYBR Green is free in solution and produces little fluorescence. The fluorescence signal is measured at the end of each elongation phase and allows the user to monitor the linear accumulation of PCR products in real-time over a range of PCR cycles. Thus, the increase in the SYBR® Green signal correlates with the amount of product amplified during PCR. The results can be displayed as an amplification plot, which reflects the change in fluorescence during cycling. Based on a threshold cycle (C_t) the initial copy number can be quantitated during real-time PCR analysis. C_t is defined as the cycle at which fluorescence is determined to be statistically significant above background. This value was used for the gene expression analysis. Since SYBR® Green binds to any dsDNA it cannot discriminate between specific products, non-specific products and primer-dimers. Since all double-stranded PCR products will contribute to the signal intensity the assay also included a melting curve analysis of the qRT-PCR products demonstrating product specificity after amplification.

For the qRT-PCR analysis 1 µl (approximately 200 ng) of total RNA was transcribed to cDNA by reverse transcription and subsequently amplified using 0.5 µM of both sense and antisense primer. Product specificity was demonstrated by melting curve analysis of qRT-PCR products. All experimental steps were performed on ice. The following pipetting scheme was used for qRT-PCR representative for one reaction:

Component	Volume
SYBR® Green Master Mix	12,5 µl
reverse transcriptase mix	1 µl
primer mix (forward and reverse)	2 µl
H_2O	8,5 µl
RNA (or H_2O as control)	1 µl

The following cycling protocol was used:

Cycles	Duration	Temperature	Procedure
1	25 min	50 °C	First-strand cDNA synthesis
1	15 min	95 °C	initial denaturation of cDNA / activation of DNA polymerase
35	15 sec	95 °C	denaturation of DNA
	20 sec	60 °C	binding of primers to DNA
	30 sec	72 °C	elongation of primers to synthesise complementary strand / measurement of fluorescence intensity
1	1 min	95 °C	denaturation of PCR products / melting curve analysis
1	30 sec	55 °C	annealing of PCR products to generate dissociation curves

The results were expressed relative to the housekeeping gene *Hmbs* (hydroxymethylbilane synthase), whose expression did not vary under the experimental conditions used. Quantification of relative RNA levels were performed using following formula of the comparative threshold cycle ($\Delta\Delta C_t$ method):

$$2^{\wedge} (C_t \text{ (housekeeping gene)} - C_t \text{ (target gene)})$$

2.2.11 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is a sensitive and specific enzyme immunoassay technique for the quantitative analysis of molecules including cytokines, chemokines, growth factors, proteases and more in an antibody sandwich format. To bind soluble factors a capture antibody is coated to a microplate. The analyte is bound to a detection antibody conjugated to horseradish peroxidase. Several washing steps remove unbound material. By adding a chromogenic substrate solution the color develops in proportion to the amount of analyte present in the sample. Subsequently the color development is stopped and absorbance is measured at 450nm.

Immobilization and release studies of proteins by surface-bound starPEG-heparin hydrogels were performed in custom-made incubation chambers that allowed

only minimal interaction of the protein solution with areas not originating from the hydrogel. 200 µl of FGF-2 and/or GDNF (0.5 or 1 µg/ml) solution were added per cm^2. After immobilization over night at 22 °C the protein solution was taken out followed by washing with PBS twice. Afterwards the proteins were allowed to release from the gels into 250 ml/cm^2 of Basic medium containing 10 % FCS or Basic medium without FCS, respectively. Samples taken at intervals were stored at -80 °C until be analyzed by ELISA. An equal volume of fresh medium was added back at each time point. Each of these solutions was collected and assayed in triplicates using an ELISA Quantikine kit for FGF-2 or E_{max}® ImmunoAssay System for GDNF according to the manufacturer's manual. Immunoassays for the quantitative analysis of FGF-2 were performed using pre-coated microplates delivered by the assay company. Within the enzyme immunoassay for the detection of GDNF non-coated Nunc-Immuno™ MaxiSorp™ plates with a high affinity to molecules with mixed hydrophilic/hydrophobic domains had to be coated prior usage.

All incubation steps were performed at room temperature if not mentioned else wise.

2.2.12 Cell counting and neurite length quantification

Cells were analyzed by conventional fluorescence microscopy and images were acquired using Leica FW4000. Image analysis, quantification of total cell number/positive cells and analysis of the axo-dendritic outgrowth of dopaminergic cells were performed with ImageJ 1.37v (developed by W. Rasband, National Institutes of Health, Bethesda, USA) or PhotoshopCS2 (Adobe). For the quantification of positive cells at least five coverslips were counted per time point and condition. Analysis of the axo-dendritic outgrowth was carried out on images showing the full extent of neurite outgrowth and on single DA neurons without any cell-cell contacts to other DA neurons. The number of neurites extending directly from the cell soma of dopaminergic cells was defined as primary neurites and quantified. At least 10 cells per time point and condition were analyzed.

2.2.13 Statistics

Statistical significance was computed using Student's t-test or one-way ANOVA and post-hoc t-test with Bonferoni adjustment using the SPSS software version 18.0 (SPSS Institute, Chicago, USA). All data are presented as mean ± S.E.M. (standard error of the mean). *P*-values < 0.05 were considered as significant.

3. Results

3.1 Extracellular matrix molecules influence growth properties of primary fetal mesencephalic cells

As components of the ECM influence several cellular processes *in vitro* and *in vivo*, the development of biomaterials mimicking regulatory characteristics of natural ECM their effects on the growth properties of primary fetal mesencephalic cells has to be investigated. Proteins of the ECM were covalently immobilized to POMA modified glass surfaces and growth properties of primary fetal mesencephalic cells isolated at E14 were analyzed after 5 days *in vitro* (DIV) using bright field microscopy and after 7 DIV with immunofluorescence staining for dopaminergic cells using fluorescence microscopy. Fetal cells were seeded in a density of 2,500 cells per mm^2 on ECM-coated glass surfaces and immunofluorescence staining observing DAergic cells was performed against tyrosine hydroxylase (TH), an enzyme catalyzing the conversion of L-tyrosine to L-DOPA the precursor of dopamine (Figure 5). Influences of ECM molecules on axo-dendritic outgrowth of DAergic cells were monitored including analysis of effects on (1) the number of neurites extending directly from cell soma (primary neurites) indicating a primary branching capability, (2) the number of all neurites indicating a branching capability of a complete neuron, and (3) the length of neurite extension suggesting a possible role in promoting neurite elongation. All quantifications were carried out using the Image J plug-in Neuron J and were compared to growth properties on glass surfaces coated with poly-D-lysine (PDL), a widely used coating agent *in vitro*. With its polycationic property, PDL encourage the interaction with anionic sites on cells promoting their attachment.

Although a lot of cells adhere to coated glass surfaces, survive and form cell extensions, differences in growth properties of fetal primary mesencephalic cells cultured on various ECM components were detected at representative bright field images after 5 DIV (Figure 5A). A homogenously cell spreading was achieved on collagen I-, fibronectin- and PDL-coated glass surfaces, whereas a formation of cell clusters was observed when cells were cultured on glass surfaces coated with tropocollagen I, collagen I/heparin, laminin and RGD.

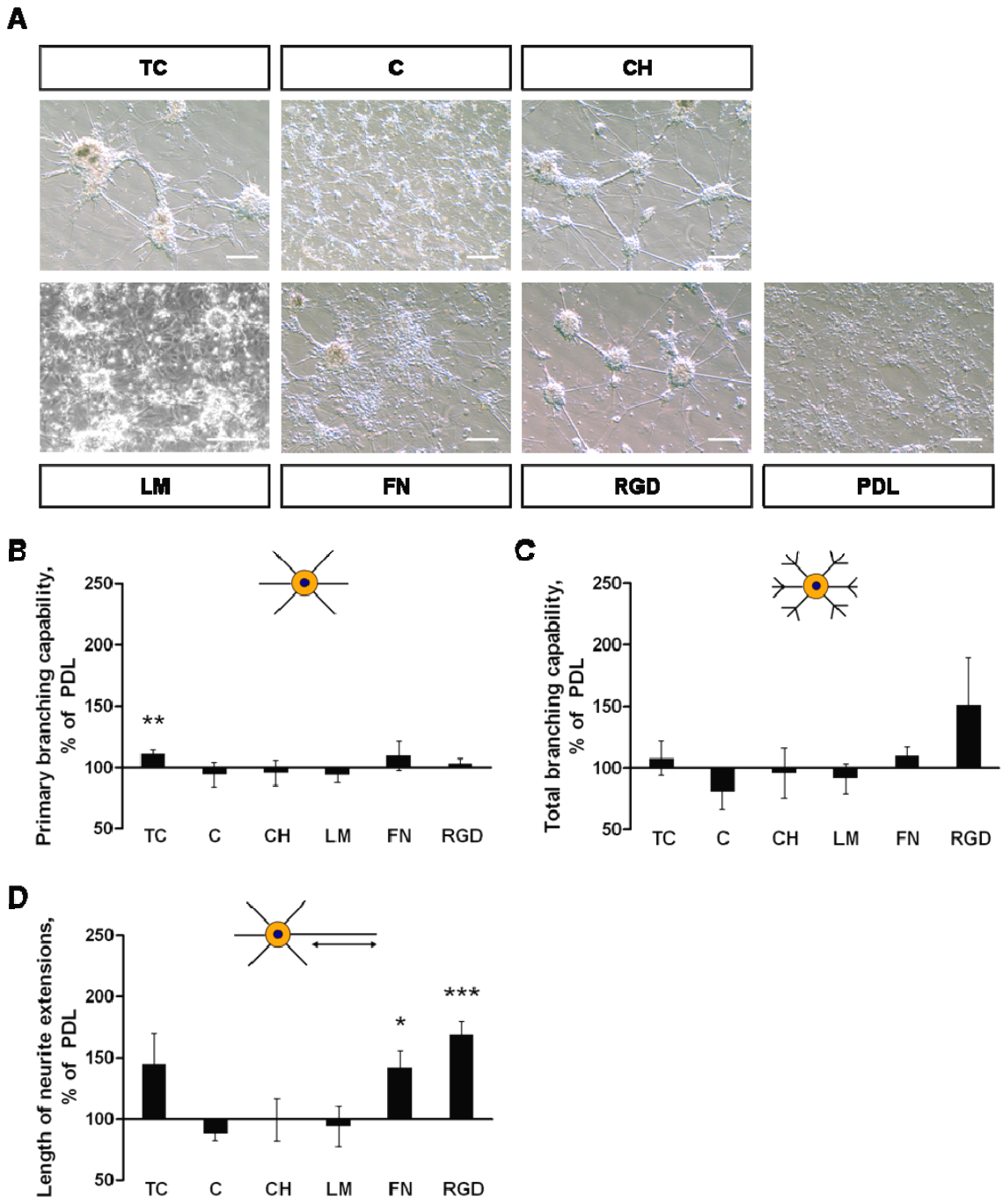

Figure 5: Influence of extracellular matrix molecules on growth properties of primary fetal mesencephalic cells.
Cells were isolated, seeded at a density of 2,500 cells per mm^2 and cultured for a period of 7 days on various ECM components. (A) Representative bright field images after 5 DIV shows different growth properties of primary fetal mesencephalic cells cultured on ECM molecules. Scale bar 200 µm. (B-D) After 7 DIV cells were immunostained for TH, axo-dendritic outgrowth of DAergic neurons compared to PDL was observed with fluorescence microscopy and quantified using Image J. Data are expressed as mean ± S.E.M. * - $P<0.05$, ** - $P<0.01$, *** - $P<0.005$, ANOVA; n=3-4. TC – tropocollagen I, C – collagen I, CH – collagen I/heparin, LM – laminin, FN – fibronectin, RGD - Arg-Gly-Asp PDL – poly-D-lysine.

In Figures 5B – D, influences of ECM molecules on axo-dendritic outgrowth of DAergic cells are displayed. A significant increase in the amount of primary neurites of DAergic cells in respect to PDL (100%) with 111±3% (F-value 0.897, *P*<0.01; ANOVA; n=4) were observed when cell were cultured on tropocollagen I. No effects of several other ECM components on the amount of primary neurites of DAergic cells in respect to PDL were observed possessing similar quantities of primary neurites (*P*>0.05; ANOVA; n=3-4).

No effect on branching of DAergic cells in respect to PDL was achieved when cell were cultured on different ECM molecules (F-value 1.664, *P*>0.05, ANOVA; n=3-4) (Figure 5C).

Fibronectin and RGD stimulate the neurite extension of dopaminergic cells significantly with an increase in neurite length of 42% (142±14%; F-value 3.856, *P*<0.05, ANOVA; n=4) and 69% (169±10%; F-value 3.856, *P*<0.005, ANOVA; n=3) compared to PDL substrate, respectively. Other ECM molecule, such as tropocollagen I, collagen I , collagen I/heparin and laminin showed now neurite promoting activity compared to PDL (P>0.05, ANOVA; n=4) (Figure 5D).

Taken together, Figure 5 demonstrates the different growth properties of primary fetal mesencephalic DAergic neurons cultured on ECM modified glass surfaces. ECM components influence cell morphology of primary fetal mesencephalic DAergic cells and it seems that fibronectin and its cell binding sequence RGD can promote axo-dendritic outgrowth of DAergic cells.

3.2 Evaluation of the starPEG-heparin hydrogel

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3.2.1 Physical/chemical properties of starPEG-heparin hydrogels

Hydrogels with differing molecular ratios of starPEG and heparin can be synthesized. By varying the amount of 4-arm starPEG and constant heparin content, different hydrogels with various network characteristics can be generated. Increasing the starPEG/heparin content will generate hydrogels with a more cross-linked polymer network [155]. An increase in the number of cross-links result in a denser network with higher rigidity (higher storage modulus) and a restricted water uptake (lower swelling) [155]. In cooperation with the research group of Prof. Carsten Werner at the

Leibniz Institute of Polymer Research Dresden hydrogels with different physical/chemical characteristics and molecular ratios of starPEG/heparin from γ=2, γ=4 and γ=6 with different cross-linking degrees, material rigidity and swelling degrees were synthesized in this work. Thereby, γ=2 indicates starPEG-heparin hydrogels with low cross-linking degrees, low storage moduli and high swelling properties, whereas γ=6 hydrogels possess much higher cross-linking and storage moduli with lower swelling degrees. Hydrogels with γ=4 show intermediate network characteristics.

3.2.2 Biofunctionalization of starPEG-heparin hydrogels

While physical characteristics of the hydrogel can be changed the heparin content remains constant. To tune the cell-adhesive properties of the hydrogel the cell adhesion mediating peptide RGD were covalently attached to the hydrogel. Covalent bonds were formed between EDC/s-NHS activated carboxylic acid groups of heparin and amino groups of lysine on the RGD. As the heparin content remain constant independent from network characteristic, the RGD amount were found to be similar [155]. Furthermore, due to the anionic character of heparin and its affinity for many soluble factors, growth factors can be incorporated independent from cross-linking degree through non-covalent interactions with heparin. Growth factors known to stimulate cellular processes can be bound to the hydrogel to further functionalize the hydrogel platform. Thus, the hydrogels display a modular system in which network characteristics can be gradually varied, while keeping the heparin content and the biomolecular functionalization constant [155]. As RGD was shown to promote neurite elongation of DAergic cells, this peptide was covalently bound to activated carboxyl groups of the heparin molecule within a starPEG-heparin hydrogel. Furthermore, growth factors, such as FGF-2 and GDNF were incorporated to the hydrogel system.

3.2.3 StarPEG-heparin hydrogels function as an efficient storage and delivery system for FGF-2 and GDNF

Since large amounts of heparin with high anionic charge density are bound to hydrogels, numerous growth factors can be incorporated and stabilized by this molecule. But not only storage of cellular effectors also their constant and

independent delivery by hydrogels is important for biomedical applications. Therefore, the analysis of immobilization and release of growth factors known to be important in treatment of PD are crucial. FGF-2 with its cell survival promoting activity and GDNF known to increase dopaminergic cell survival and to stimulate axo-dendritic outgrowth were immobilized to star-PEG heparin hydrogels. Either single protein solution with 1 µg/ml protein or incubation solutions with a combination of both factors (0.5 µg/ml or 1 µg/ml each) based on the concentrations used for *in vitro* studies. Protein immobilization and release were performed in custom-made immobilization chambers minimizing protein interaction with surfaces of the device. Besides analyzing whether the hydrogel can be used as an efficient storage and delivery system for various amounts of growth factors, the evidence that uptake and release of GDNF occur independently from cross-linking degree of the hydrogel were submitted. In general, the release was carried out in the same cell culture media as used for *in vitro* studies of primary fetal mesencephalic cells if not indicated elsewise. The amount of protein was quantified by enzyme-linked immunosorbent assays (ELISA).

To investigate the uptake and release of growth factors from hydrogels either 1 µg/ml FGF-2 and 1µg/ml GDNF as single components or 0.5 µg/ml and 1 µg/ml as factor combinations were immobilized to gels with low cross-linking degree, γ=2 (Figure 6).

Figure 6A shows the immobilization of similar quantities for both proteins either as single factors or as factor combinations. For single factors 256±1 ng/scaffold for FGF-2 and 258±1 ng/scaffold for GDNF (P>0.05, Student's t-test; n=3) could be immobilized from a solution of 1 µg/ml protein. Similar amounts of growth factors immobilized from a solution containing both proteins in a concentration of 1 µg/ml each were found (FGF-2: 257±1 ng/scaffold; GDNF: 260±2 ng/scaffold) (P>0.05, Student's t-test; n=3) indicating that the two factors do not affect each other while interacting with the hydrogel matrix. Moreover, the same protein amount was immobilized either as single factor or as factor combination (FGF-2 single: 256± 1 ng/scaffold and in combination 257±1 ng/scaffold (P>0.05, Student's t-test; n=3)) (GDNF single: 258±1 ng/scaffold and in combination 260±2 ng/scaffold (P>0.05, Student's t-test; n=3)). Furthermore, a linear correlation between the concentration of

incubation solution and the amount of immobilized protein were observed. When a solution of 0.5 µg/ml of both proteins were incubated with the hydrogel 120±2 ng/scaffold FGF-2 and 130±1 ng/scaffold GDNF were immobilized (P<0.005, Student's t-test; n=3). Notably, the amount of immobilized FGF-2 when 0.5 µg/ml was used is more than twice lower compared to the protein amount when 1 µg/ml FGF-2 is loaded. For GDNF the amount of immobilized protein is almost twice higher when the incubation solution exhibits a twice higher concentration.

Subsequently, the diffusion-based release of FGF-2 and GDNF from hydrogels was studied. Figures 6B and C illustrate the cumulative release of FGF-2 alone or in combination with GDNF over a period of 7 days. An initial burst release was observed within the first 24 hours followed by a continuous, slow release over time. Regardless, whether factors were immobilized alone or in combination the released protein amount were similar. For an immobilization with 1 µg/ml FGF-2 53±1 ng/scaffold and 50±3 ng/scaffold of released protein were observed for single or combined FGF-2, respectively after 168 h (P>0.05, Student's t-test, n=3) (Figure 6B left). For GDNF a overall protein amount of 47±2 ng/scaffold for single factor and 37±6 ng/scaffold for the combination were analyzed (P>0.05, Student's t-test, n=3), whereas half of the protein amount is released, when only half of the quantity is immobilized (FGF-2/0.5 µg/ml: 30±4 ng/scaffold; GDNF/0.5 µg/ml: 19±2 ng/scaffold; P>0.05, Student's t-test, n=3) (Figure 6B right). Furthermore, a linear correlation between the amount of protein used for immobilization and the quantities being released were investigated for factor combinations (FGF2: 30±4 ng/scaffold for 0.5 µg/ml and 50±3 ng/scaffold for 1µg/ml; GDNF: 19±2 ng/scaffold for 0.5 µg/ml and 37±6 ng/scaffold for 1.0 µg/ml).

For an immobilization of 1 µg/ml FGF-2 as single factor or in combination huge protein amounts were released within the first 24 hours (FGF-2 single: 20±0.2% and in combination: 18±1% (P>0.05, Student's t-test; n=3); GDNF single: 17±1% and in combination: 14±2% (P>0.05, Student's t-test; n=3). After 7 days the amount of released protein is only slightly increased (FGF-2 single: 21±0.2% and in combination: 20±1% (P>0.05, Student's t-test; n=3); GDNF single: 18±1% and in combination: 15±2% (P>0.05, Student's t-test; n=3) indicating that huge quantities of growth factors still remain in the hydrogel matrix.

In summary, the use of star-PEG-heparin hydrogels as efficient storage and delivery system were demonstrated. Furthermore, the independent loading and release of two different factors in two different concentrations could be demonstrated.

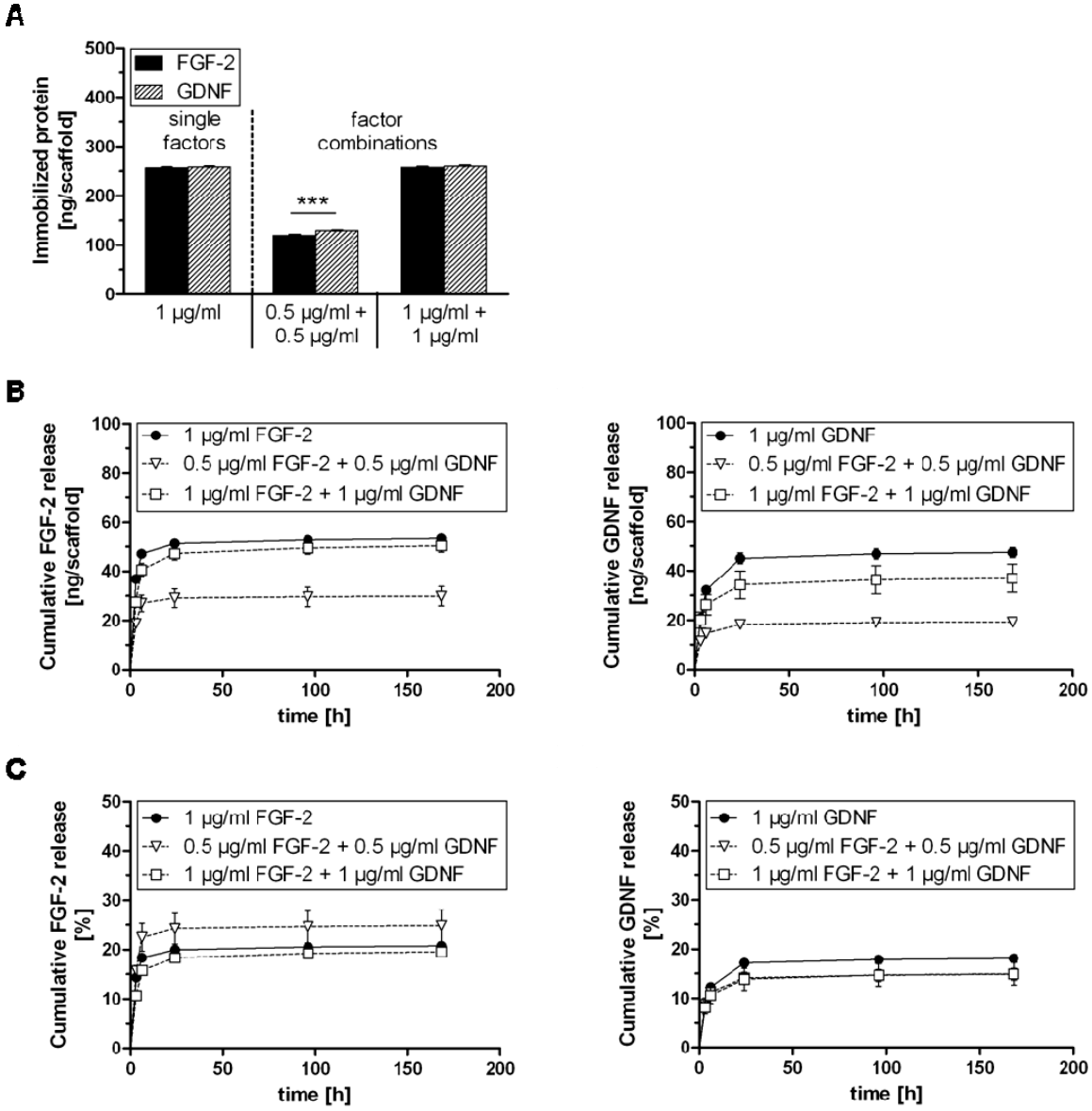

Figure 6: Uptake and release of FGF-2 and GDNF separately or in combination with each other.
Hydrogels were immobilized with single FGF-2 or GDNF or combinations of both. The protein amount was quantified after several time points using ELISA. (A) The amount of electrostatically bound FGF-2 and/or GDNF per scaffold is shown. (B-C) The plots display the cumulative amount of bound protein released by the hydrogels and the cumulative percentage of released protein, respectively for duration of 7 days. Dashed lines indicate the release in case of factor combinations, whereas continuous lines denote the release in case of single factors. Data are expressed as mean ± S.E.M. *** - $P<0.005$; Student's t-test, n=3.

Recently, it was shown that the release of FGF-2 is different when using medium free of serum proteins compared to medium with bovine serum albumine (BSA). Roughly 10 % more FGF-2 could be obtained in serum conditions compared to medium without BSA. Therefore, investigating the release of GDNF is of quite big importance due to the use of serum-free medium to culture NSCs. In detail, 1 µg/ml GDNF were immobilized to gels with low cross-linking degree (γ=2) and the release were either performed in serum media containing 10 % FCS used for primary fetal mesencephalic culture or in serum-free media used for fetal mesencephalic NSC culture (Figure 7).

In both conditions an initial burst release was shown and the release slowly continued over the period of time (Figure 7A). As expected, the overall release of GDNF in serum containing medium was found to be much higher than the release in serum-free medium (serum-containing medium: 47±2 ng/scaffold; serum-free medium: 4.3±0.3 ng/scaffold; $P<0.005$, Student's t-test, n=3). Roughly 10 % less GDNF under SFM condition were quantified compared to SM settings (serum-containing: 18±1 %; serum-free medium: 1.6± 0.1%; $P<0.005$, Student's t-test, n=3) (Figure 7B).

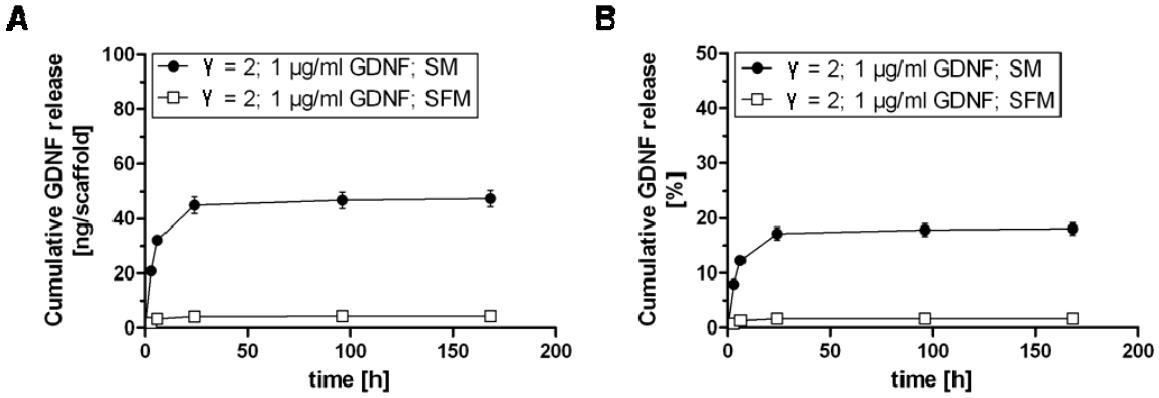

Figure 7: Uptake and release of GDNF in dependence on the release medium.
Hydrogels were immobilized with 1 µg/ml GDNF and protein was released into serum-free (SFM) or serum containing medium (SM) with 10 % FCS. The protein amount was quantified at several time points using ELISA. (A-B) Plots show the cumulative protein amount per scaffold and the cumulative percentage of released protein, respectively. Data are expressed as mean ± S.E.M., Student's t-test, n=3.

3.2.4 Fetal mesencephalic neural stem cells respond to mechanical and biomolecular properties of starPEG-heparin hydrogels

Fetal mesencephalic NSCs are promising cell source for neural transplantation in PD. To investigate their survival and differentiation properties on different settings of hydrogels, cells were isolated at E14 and expanded as suspension cultures for one week prior use. Cells were transferred to differentiation media with 5 mM forskolin and 100 pg/ml interleukin (IL)-1β and seeded on hydrogels. Three different biohybrid gels with an increasing molecular ratio of star-PEG to heparin from γ=2, γ=4 and γ=6 were used to form hydrogels with different cross-linking degrees, storage moduli and swelling properties. Each hydrogel with defined cross-linking degree were used without biomolecular functionalization and with covalent attachment of cell adhesion peptide RGD and FGF-2. Both factors were tested alone or in combination. Thus, the influence of network characteristics and biomolecular functionalization on fetal mesencephalic NSCs could be investigated.

Figure 8 displays the interaction of NSCs with hydrogels of various network characteristics and biomolecular functionalizations in a two-dimensional cell culture after 5 DIV. NSCs grew even on unmodified hydrogels with the best cell survival obtained on hydrogels with the highest stiffness. Regardless of biomolecular functionalization, the hydrogel with the highest cross-linking degree showed highest cell survival. Biomolecular functionalization with RGD and FGF-2 or the combination of both had very different effects on NSCs. Cells cultured on plain gels and on FGF-2 loaded gels showed a “neurosphere”-like aggregates with no speading of cells outside of the “neurosphere”. On FGF-2 loaded hydrogels “neurospheres” appeared much larger indicating a higher cell survival. Attachment of integrin binding ligand RGD led cells spread on hydrogels without “neurosphere”-like formation. For hydrogels with RGD and FGF-2 loading a tremendous increase of cell survival were observed.

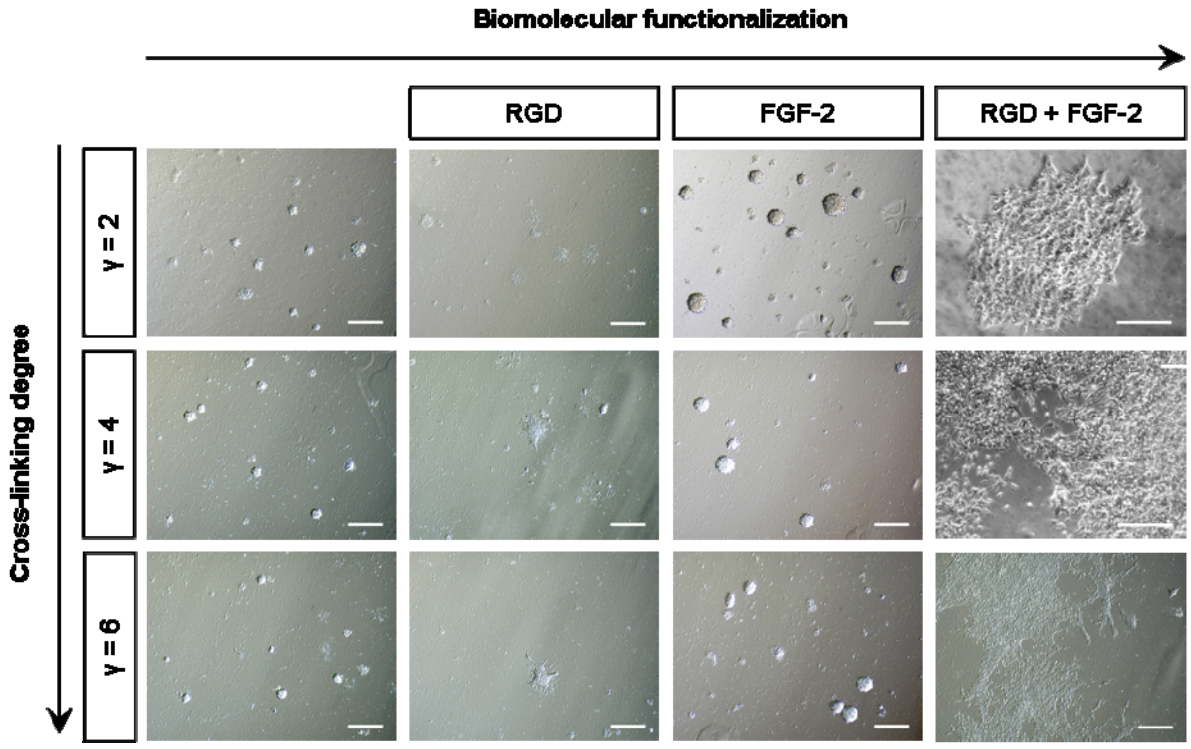

Figure 8: Interaction of various starPEG-heparin hydrogels and fetal mesencephalic neural stem cells (NSCs).
Cells were isolated, expanded in suspension culture for 7 days, transferred to differentiation media and seeded on hydrogels. Representative bright field images show the different growth properties of fetal mesencephalic NSCs after 5 DIV. Scale bar, 200 µm.

To investigate the differentiation behavior of NSCs cultured on various gel types, cells were stained for the neural stem cell marker Nestin as well as for Tuj1, which is expressed in immature neurons and their committed progenitors. Cell nuclei were counterstained with Hoechst 33342, a DNA intercalating agent. Figure 9 shows representative immunofluorescence images on low cross-linked hydrogels (γ=2). Similar results were found on more cross-linked hydrogels (γ=4, γ=6). NSCs on plain hydrogels showed only poor differentiation, whereas incorporation of the cell adhesion mediating peptide RGD led to differentiation of NSCs into neurons. Nearly a homogenously Nestin staining were observed in “neurosphere”-like formations on FGF-2 loaded gels with sporadic neuronal differentiation and higher cell survival. By combining RGD peptide and FGF-2 to hydrogels many $Nestin^{+}$ NSCs and $Tuj1^{+}$ neurons with mature axo-dendritic arborization were investigated with an tremendous increase in cell survival.

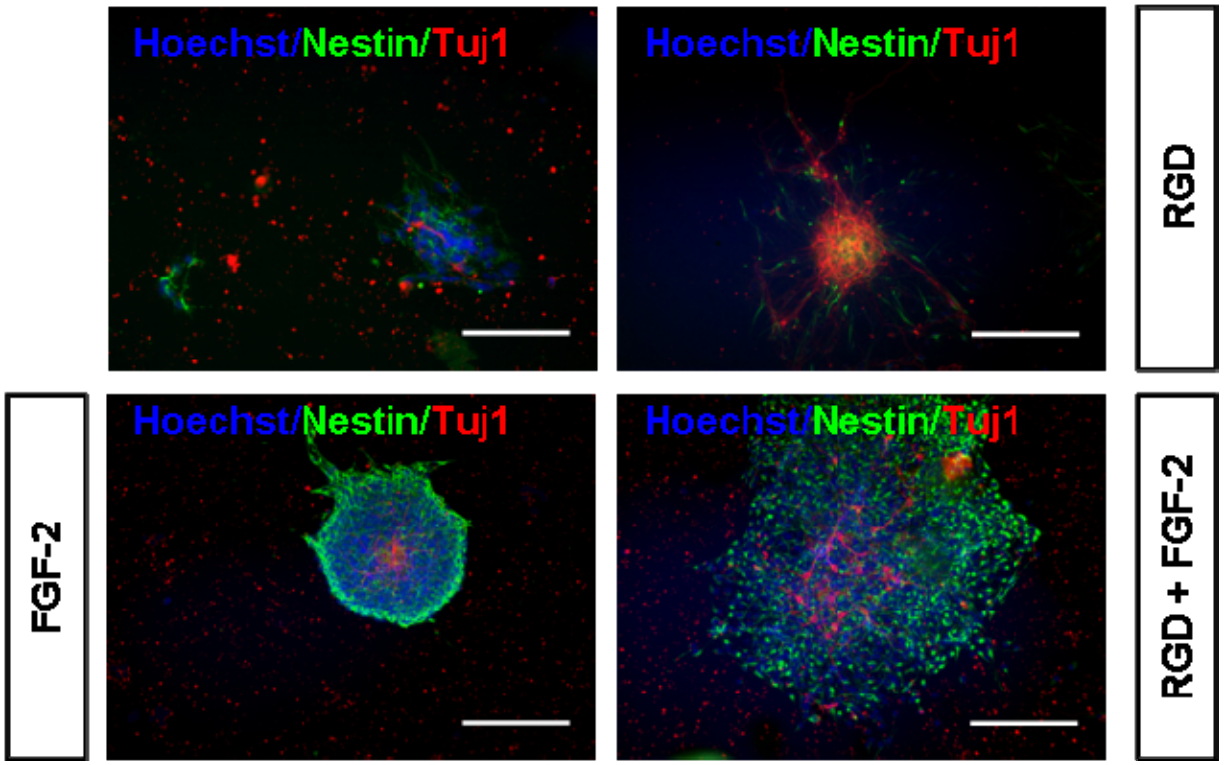

Figure 9: Influence of various hydrogels on survival and differentiation of fetal mesencephalic neural stem cells (NSCs).
Cells were isolated and expanded in suspension culture for 7 days. After transferring into differentiation media cells were seeded on hydrogels and cultured for 7 DIV. Representative images of the immunoreaction to the neural stem cell marker Nestin and immature nerve cell marker Tuj1 on hydrogels with the lowest cross-linking degree (γ=2) are shown. Scale bar, 200 µm.

3.2.5 Network structure and biomolecular functionalization of starPEG-heparin hydrogels influence the cell survival and axo-dendritic outgrowth of primary fetal mesencephalic cells

To increase the viability and maturity of cells, the hydrogels were modified with the cell adhesion peptide RGD and loaded with the soluble mitogen FGF-2. Additionally, the influence of different structural and mechanical characteristics on the interaction with primary fetal mesencephalic cells was evaluated. The cells were cultured on a set of different biohybrid gels with an increasing molecular ratio of star-PEG to heparin from γ=2, γ=4 and γ=6. Each hydrogel with defined cross-linking degree were used without biomolecular functionalization and with covalent attachment of cell adhesion peptide RGD and FGF-2. Both factors were tested alone or in combination.

Thus, the influence of network characteristics and biomolecular functionalization on primary fetal mesencephalic cells could be investigated. Primary fetal mesencephalic cells were isolated at E14 and seeded with a cell density of 2,500 cells per mm^2 on biohybrid gels.

To investigate the influence of hydrogels with various biophysical and biomolecular characteristics the 2-dimensional cell culture was monitored using bright field microscopy after 5 DIV (Figure 10). Different growth behavior was observed depending on cross-linking degree and biomolecular functionalization. Plain gels without biomolecular modification showed only low cell survival, whereas the attachment of RGD to the hydrogel led to a slight increase in cell survival. With the incorporation of FGF-2 the cell survival increased dramatically with exception for the hydrogel with the highest cross-linking degree. High survival rates were also obtained with combination of covalently attached RGD and heparin-conjugated FGF-2 even for the highest cross-linking network structure.

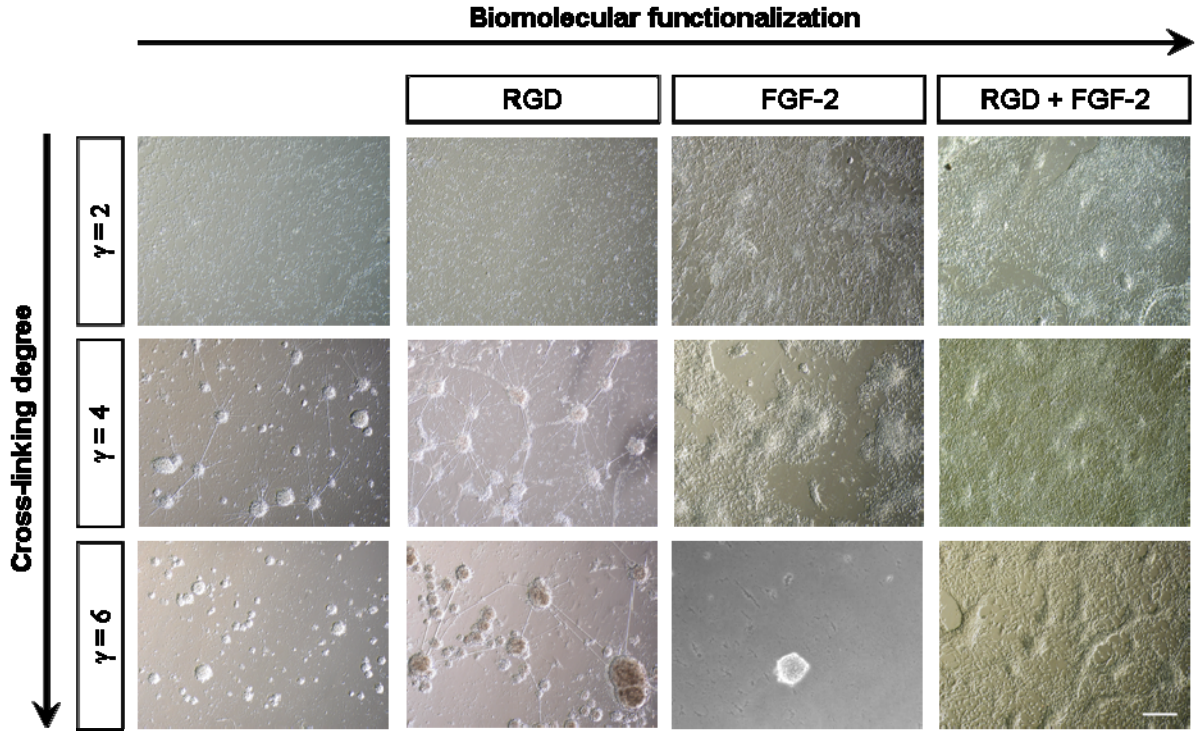

Figure 10: Interaction of various starPEG-heparin hydrogels and primary fetal mesencephalic cells.
Cells were isolated and seeded at a density of 2,500 cells per mm^2. Representative bright field images show different growth properties of primary fetal mesencephalic cells after 5 DIV. Scale bar, 200 µm.

In order to study the survival of neuronal cell types after 7 DIV cells were stained for the mature neuronal marker MAP2 as well as for TH identifying DAergic cells. Cell nuclei were counterstained with Hoechst 33342, a DNA intercalating agent. Representative immunofluorescence images in Figure 11 show the immunoreactivity to MAP2 and TH on low cross-linked hydrogels (γ=2). Similar result were found for more cross-linked hydrogels (γ=4, γ=6). Plain and RGD-modified gels showed only a low cell survival with a low number of $MAP2^+$ cells per mm^2, whereas on FGF-2-loaded hydrogels with or without RGD the cell number and the amount of $MAP2^+$ cells per mm^2 were increased.

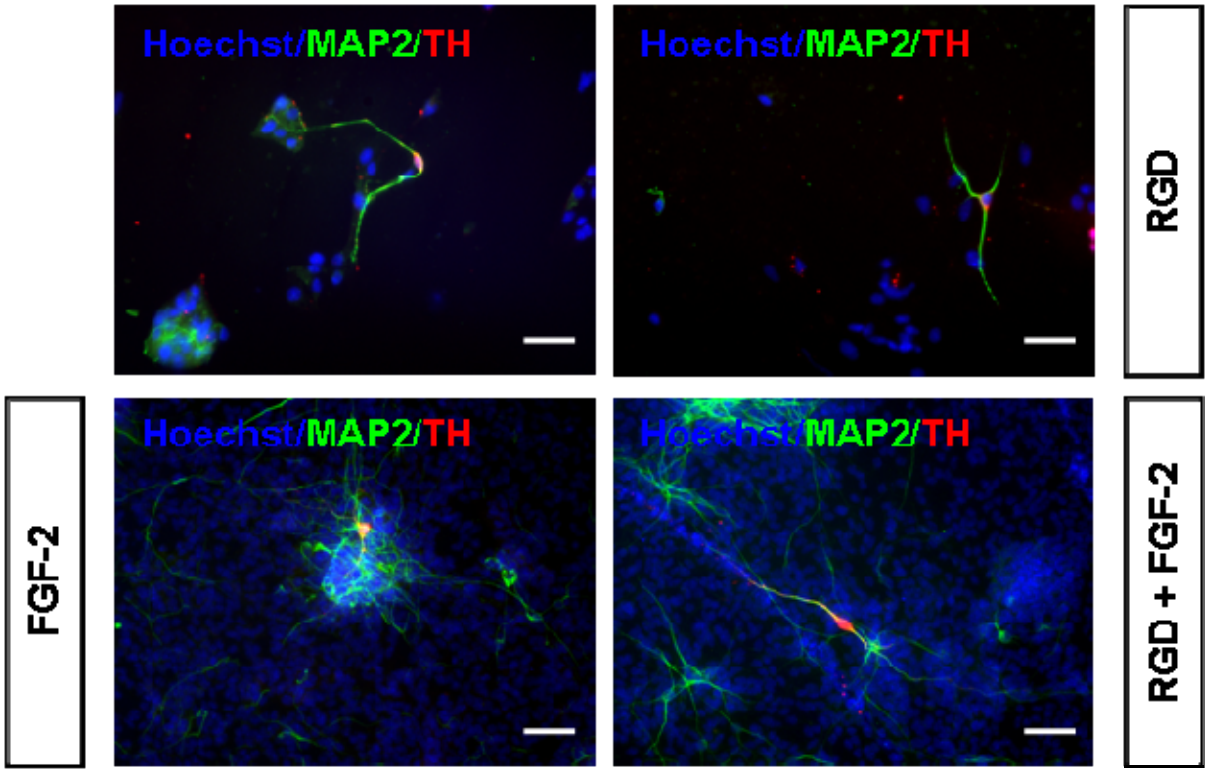

Figure 11: Interaction of starPEG-heparin hydrogels with neuronal cell types of primary fetal mesencephalic cells.
Cells were isolated and seeded at a density of 2,500 cells per mm^2. After 7 DIV cells were stained for the mature neuronal marker MAP2 and the marker for dopaminergic cells TH. Cell nuclei were counterstained with Hoechst 33342. Representative images of the immunoreaction to the mature neuronal marker MAP2 and the marker for dopaminergic cells TH on hydrogels with the lowest cross-linking degree (γ=2) are shown. Scale bar, 50 µm.

To determine the cell survival on hydrogels with different chemical/physical characteristics (γ=2, γ=4, γ=6) and biomolecular functionalization (pure gel, modified with RGD, loaded with FGF-2 or with a combination of RGD+FGF-2) $Hoechst^+$ cells,

MAP2$^+$ cells as well as TH$^+$ cells were quantified in respect to mm^2 after 7 DIV (Figure 12). The number of Hoechst $^+$ cells indicates the total cell number.

Figure 12A displays the quantitative analysis of the total cell number. On hydrogels with low network density (γ=2) significant more cells were quantified on hydrogels with FGF-2 and RGD+FGF-2 compared to pure and/or RGD modified hydrogels with the same network characteristics (FGF-2: 5702±500 cells/mm^2 (γ = 2) and RGD+FGF-2: 6016±254 cells/mm^2 both compared to pure gel 39±7 cells/mm^2 and both compared to RGD 40±8 cells/mm^2; F-value 136.243, *P*<0.005, ANOVA; n=3). A similar survival pattern of Hoechst$^+$ cells were achieved on FGF-2 and RGD+FGF-2 hydrogels with middle network density (γ=4) compared to pure and/or RGD modified hydrogels with the same cross-linking degree (FGF-2: 4086±602 cells/mm^2 and RGD+FGF-2: 6897±372 cells/mm^2 both compared to pure gel 30±10 cells/mm^2 and both compared to RGD 192±19 cells/mm^2; F-value 136.243, *P*<0.005, ANOVA; n=3). RGD+FGF-2 loaded hydrogels with highest cross-linking degree (γ=6) displayed the highest cell survival compared to pure gel and RGD modified hydrogels with the same network density (RGD+FGF-2: 7407±254 cells/mm^2 compared to pure gel 15 ± 9 cells/mm^2 and compared to RGD modified gel 137±40 cells/mm^2;F-value 136.243, *P*<0.005, ANOVA; n=3). Interestingly, only few cells survived on FGF-2 loaded hydrogels with highest cross-linking degree as also observed in the bright field images. The combination of RGD and FGF-2 to this gel type could erase this inhibitory effect and a significant increase of cell survival was quantified (RGD+FGF-2: 7407±254 cells/mm^2 compared to FGF-2 (10±5 cells/mm^2; F-value 136.243, *P*<0.005, ANOVA; n=3). Furthermore, the low cell survival on FGF-2 loaded hydrogels with highest cross-linking degree could be increased when cells were cultivated on lower cross-linked gels (FGF-2: 10±5 cells/mm^2 (γ=6) compared to 5702±500 cells/mm^2 (γ=2) and compared to 4086±602 cells/mm^2 (γ=4); F-value 136.243, *P*<0.005, ANOVA; n=3). On other hydrogel settings only low cell survival were achieved independent on cross-linking degree or biomolecular functionalization. Taken together, a high cell survival could be achieved on hydrogels with heparin-conjugated FGF-2 except for hydrogels with the highest cross-linking degree. For the combination of both factors RGD and FGF-2 high cell survival rates could be obtained even for highly cross-linked hydrogels.

Similar results were achieved for MAP2-immunoreactivity of mesencephalic cells grown on hydrogels (Figure 12B). Significant more $MAP2^+$ cells were obtained on FGF-2 and RGD+FGF-2 loaded hydrogels with middle cross-linking degree when compared to pure gels with same network characteristic (FGF-2: 73±10 cells/mm^2 (γ=4), RGD+FGF-2: 76±17 cells/mm^2 (γ=4) both compared to pure gel 3±1 cells/mm^2 (γ= 4), F-value 8.972, *P*<0.05, ANOVA; n=3). Furthermore, a significant increase of mature neuronal cells were obtained on hydrogels with highest network density compared to pure gel, RGD modified and FGF-2 loaded hydrogels with the same cross-linking degree (RGF+FGF-2: 105±30 cells/mm^2 (γ=6) compared to pure gel 2±2 cells/mm^2 (γ=6), RGD modified gel 16±6 cells/mm^2 (γ=6) and FGF-2 loaded gel 0.1±0.1 cells/mm^2 (γ=6), F-value 8.972, *P*<0.005, ANOVA; n=3). Hydrogels loaded with FGF-2 and middle cross-linking degree also promote the survival of $MAP2^+$ cells compared to hydrogel with the same biomolecular functionalization but higher cross-linking degree (FGF-2: 73±10 cells/mm^2 (γ=6) compared to 0.9±0.9 cells/mm^2 (γ=6); F-value 8.972, P<0.05; ANOVA; n=3). To conclude, hydrogels with RGD+FGF-2 incorporation and middle and high network density as well as FGF-2 loaded gel with middle cross-linking degree were the best conditions for the survival of mature neuronal cells obtained so far.

Figure 12C displays the survival of dopaminergic cells (TH^+) on different settings of hydrogels. Significant more TH^+ cells survive when grown on RGD modified hydrogels with low or middle cross-linking degree compared to pure gel with the same network characteristic (RGD: 0.3±0.01 (γ=2) compared to pure gel 0.1±0.05 (γ=2); F-value 11.313, *P*<0.05, ANOVA; n=3) (RGD: 0.5±0.03 (γ=4) compared to pure gel 0.1±0.03 (γ=4); F-value 11.313, *P*<0.005, ANOVA; n=3). The incorporation of FGF-2 or the combination of RGD+FGF-2 to hydrogels with middle network density significantly decrease the number of TH^+ cells compared to RGD modified hydrogel with the same cross-linking degree (FGF-2: 0.2±0.07 (γ=4) and RGD+FGF-2: 0.2±0.09 both compared to RGD modified gel; F-value 11.313, *P*<0.01, ANOVA; n=3). But RGD incorporation to hydrogels with high cross-linking degree significantly decrease the amount of dopaminergic cells compared to hydrogels with the same biomodification but middle network density (RGD: 0.1±0.02 (γ=6) compared to 0.5±0.03 (γ=4), F-value 11.313, *P*<0.005, ANOVA; n=3). In other conditions the survival of dopaminergic cells were not affected neither by network characteristic nor

by biomolecular functionalization. To summarize, RGD modified hydrogels with middle cross-linking degree seems to be a condition, where dopaminergic cells survive most.

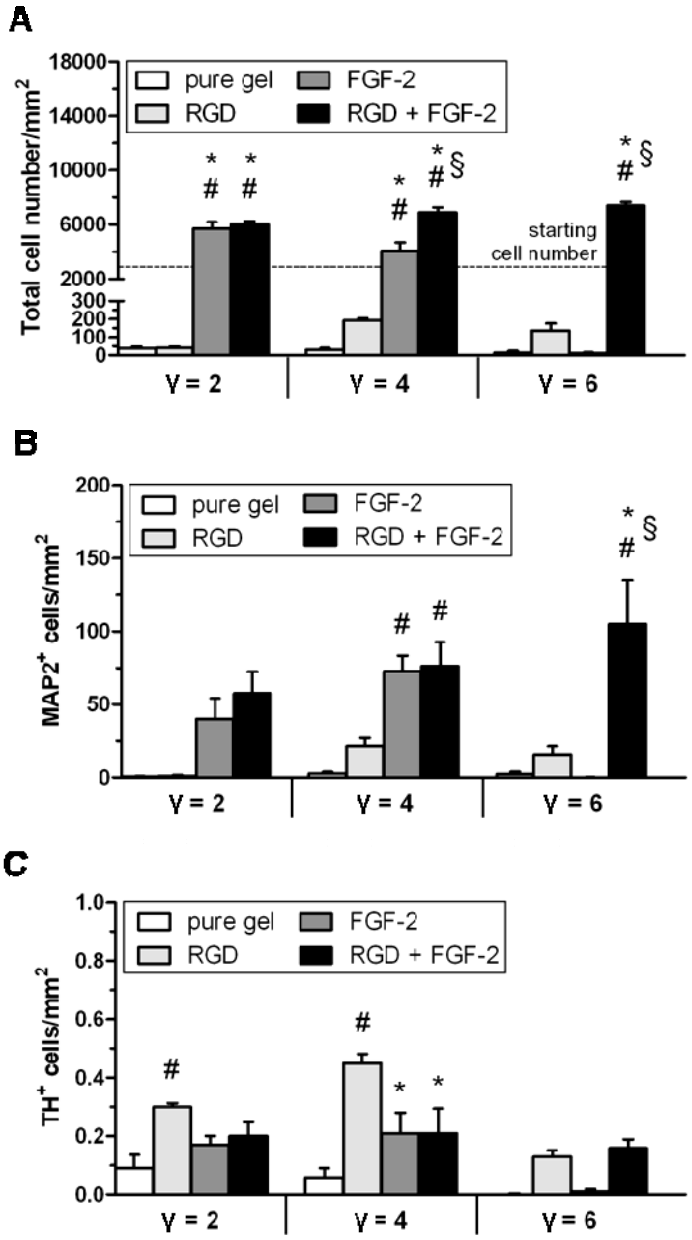

Figure 12: Interaction of starPEG-heparin hydrogels with neuronal cell types of primary fetal mesencephalic cells.
Cells were isolated and seeded at a density of 2,500 cells per mm^2. After 7 DIV cells were stained for the mature neuronal marker MAP2 and the marker for dopaminergic cells TH. Cell nuclei were counterstained with Hoechst 33342. Quantitative analysis of total cell number, MAP2 and TH displays a dramatically increase in total cell number and MAP2$^+$ neuronal cells cultured on FGF-2-loaded hydrogels, whereas RGD-modified hydrogels led to higher survival of TH$^+$ cells. Data are expressed as mean ± S.E.M. For simplification values are indicated as significant within the gel type and strength of significance are shown for P<0.05. # - significant compared to pure gel, * - significant compared to RGD modified gel, § - significant compared to FGF-2 loaded gel; ANOVA; n=3.

Besides investigating the cell survival of primary fetal mesencephalic cells cultured on the various hydrogel settings, the influence of these hydrogels on the axo-dendritic outgrowth of dopaminergic cells is indispensable for an application *in vivo*. Studying axo-dendritic outgrowth of DAergic neurons were performed similar to the analysis on ECM compounds. In detail, primary fetal mesencephalic cells were isolated at E14, seeded in a density of 2,500 cells per mm^2 on three gel types with different cross-linking degrees (γ=2, γ=4, γ=6) with/without functionalization with the cell binding ligand RGD and/or the growth factor FGF-2. After 7 DIV cells were stained for DAergic cells (TH^+ cells) and analysis were performed using a fluorescence microscope. For the analysis of axo-dendritic outgrowth of DAergic cells, 10 cells per condition were quantified using the Image J plug-in Neuron J. Hydrogels with less than 10 dopaminergic cells were indicated as not representative and thus excluded from quantification. Results are displayed in Figure 13. In all conditions three independent experiments were analyzed (n = 3).

DAergic cells grown on hydrogels with low and middle network density showed independent on their cross-linking degree and biomolecular functionalization similar amounts of primary neurites of dopaminergic cells (Figure 13A). As no DAergic cells could be quantified on FGF-2 loaded hydrogel with highest cross-linking degree, cells on hydrogels with the same biomolecular functionalization but lower cross-linking degree were found to be significant compared to stiff hydrogels (FGF-2: 2.5±0.1 (γ=2) compared to 0.0±0.0 (γ=6), F-value 7.590, $P<0.05$, ANOVA; n=3; 3.2±0.3 (γ=4) compared to 0.0±0.0 (γ=6), F-value 7.590, $P<0.005$, ANOVA; n=3). Furthermore, on hydrogels with highest cross-linking degree a significant increase in branching of the primary neurites were obtained for RGD modified gels and hydrogels containing both RGD and FGF-2 compared to pure gel and FGF-2 loaded hydrogel due to the less survival of DAergic cells under these conditions (RGD: 3.0±0.2 (γ=6) compared to pure gel 0.0±0.0 (γ=6) and to FGF-2 0.0±0.0 (γ=6); F-value 7.590, $P<0.005$, ANOVA; n=3; RGD+FGF-2: 2.8±0.2 (γ=6) compared to pure gel 0.0±0.0 (γ=6) and FGF-2 0.0±0.0 (γ=6); F-value 7.590, $P<0.01$, ANOVA; n=3). In summary, DAergic neurons showed nearly the same capability for primary branching regardless of the cross-linking degree and biomolecular functionalization with exceptions for the plain hydrogel and the FGF-2 incorporated hydrogel with the highest cross-linking degree. For these gel types not enough DAergic cells for quantification were available.

That hydrogel settings also influence the total branching of DAergic cells is displayed in Figure 13B. Significantly higher branching of DAergic cells were obtained on FGF-2 loaded hydrogels with middle cross-linking degree compared to hydrogels with low and high network density (FGF-2: 12±2 (γ=4), compared to 4.9±0.3 µm (γ=2); F-value 15.392, *P*<0.005, ANOVA; n=3 and compared to 0.0±0.0 (γ=6); F-value 15.392, *P*<0.005, ANOVA; n=3). Furthermore, dopaminergic cells on RGD modified hydrogels with middle network density promoted significantly the branching of dopaminergic cells compared hydrogel with low network density (RGD: 13±1 (γ=4) compared to 5.3±0.4 (γ=2); F-value 15.392, P<0.005, ANOVA; n=3). Incorporation of RGD and FGF-2 to hydrogels with middle cross-linking degree led to a significant decrease in DAergic branches compared to RGD modification alone (RGD+FGF-2: 6.3±1.0 (γ=4) compared to RGD 13±1 (γ=4), F-value 15.392, *P*<0.05, ANOVA; n=3) A significant increase in the total amount of branches of DAergic cells were obtained on hydrogels with middle cross-linking degree when RGD or FGF-2 is incorporated compared to pure gel with the same network characteristic (RGD: 13± 1 (γ=4) compared to pure gel 3.5±2.0 (γ=4), F-value 15.392, *P*<0.005, ANOVA; n=3); FGF-2: 12±2 (γ=4) compared to pure gel 3.5±2.0 (γ=4), F-value 15.392, *P*<0.005, ANOVA; n=3). No quantification of neurite branching could be performed on FGF-2 loaded hydrogel with highest cross-linking degree due to the less cell survival of DAergic cells under these conditions. At this cross-linking degree a significant increase of the total amount of neurite branches were obtained for RGD modified gels and hydrogels containing both RGD and FGF-2 compared to pure gel and FGF-2 loaded gels (RGD: 11±1 (γ=6) compared to pure gel 0.0±0.0 µm (γ=6) and to FGF-2 0.0±0.0 (γ=6), F-value 15.392, *P*<0.005, ANOVA; n=3; RGD+FGF-2: 7.8±1.0 (γ=6) compared to pure gel 0.0±0.0 (γ=6) and FGF-2 0.0±0.0 (γ=6); F-value 15.392, *P*<0.005, ANOVA; n=3). In all other conditions the total branching capability of DAergic cells were detected to be similar to hydrogels with the same cross-linking degree or biomolecular functionalization. Taken together, the strongest effect on the branching of dopaminergic cells were obtained when cells were cultured on RGD-modified and FGF-2 bound hydrogels with intermediate cross-linking degree.

The neurite length of DAergic cells were also affected by hydrogel settings (Figure 13C). A significant increase in the neurite length of dopaminergic

cells were obtained on hydrogels with middle cross-linking degree when RGD or FGF-2 is incorporated compared to pure gel (RGD: 861±46 µm (γ=4) compared to pure gel 162±91 µm (γ=4); F-value 12.227; *P*<0.01, ANOVA; n=3; FGF-2: 1191±259 µm (γ=4) compared to pure gel 162±91 µm (γ=4), F-value 12.227, *P*<0.005, ANOVA; n=3). Similar to the analysis of the primary neurites and the total amount of branches the neurite length of dopaminergic cells could not be quantified on FGF-2 loaded hydrogel with highest cross-linking degree due to their less cell survival under these conditions. At this cross-linking degree a significant increase in neurite length were obtained for RGD modified gels and hydrogels containing both RGD and FGF-2 compared to pure gel and FGF-2 loaded gels (RGD: 890±111 µm (γ=6) compared to pure gel 0.0±0.0 µm (γ=6) and to FGF-2 0.0±0.0 µm (γ=6); F-value 12.227, *P*<0.005, ANOVA; n=3) (RGD+FGF-2: 754±124 µm (γ=6) compared to pure gel 0.0±0.0 µm (γ=6) and FGF-2 0.0±0.0 µm (γ=6), F-value 12.227, *P*<0.005, ANOVA; n=3). Significantly longer neurites were also obtained on FGF-2 loaded hydrogels with middle cross-linking degree compared to hydrogels with low and high network density (FGF-2: 1191±259 µm (γ=4) compared to 441±13 µm (γ=2) and compared to 0.0±0.0 µm (γ=6); F-value 12.227, *P*<0.005, ANOVA; n=3)). In all other conditions dopaminergic cells showed similar neurite length to hydrogels with the same cross-linking degree or biomolecular functionalization. In summary, DAergic neurons showed nearly the same neurite length elongation regardless of the cross-linking degree and biomolecular functionalization with exceptions for the plain hydrogel and the FGF-2 incorporated hydrogel with the highest cross-linking degree. For these gel types not enough dopaminergic cells for quantification were available. The most promising condition in promoting the neurite length was obtained on hydrogels with middle cross-linking degree and FGF-2 incorporation.

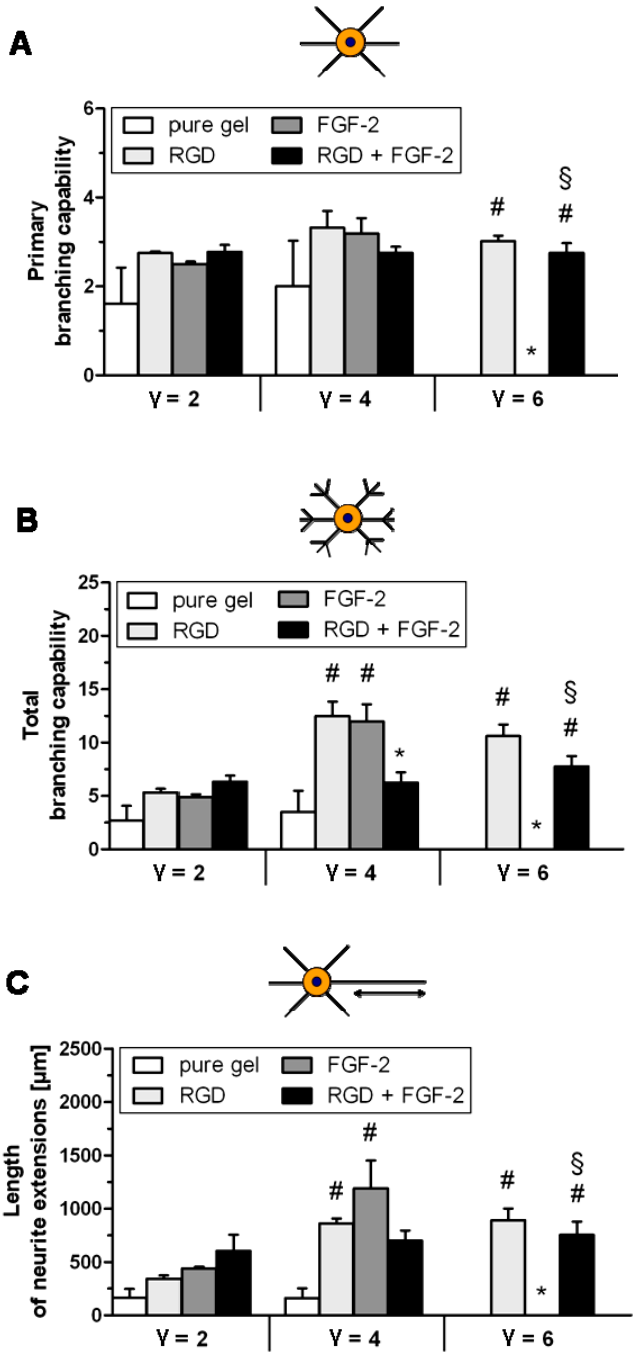

Figure 13: Influence of various starPEG-heparin hydrogels on axo-dendritic outgrowth of primary fetal mesencephalic cells.
Cells were isolated and seeded at a density of 2,500 cells per mm^2. After 7 DIV cells were stained for the marker for dopaminergic cells TH, axo-dendritic outgrowth were observed using fluorescence microscopy and cells were analyzed via Image J. (A-C) Different parameters of axo-dendritic outgrowth were analyzed including number of primary neurites extending directly from the cell soma, number of all neurites indicating neuronal branching capability and length of neurite extension suggesting a possible role in promoting neurite elongation. Data are expressed as mean ± S.E.M. For simplification values are indicated as significant within the same gel type and strength of significance are shown for $p \leq 0.05$. # - significant compared to pure gel, * - significant compared to RGD modified gel, § - significant compared to FGF-2 loaded gel.; ANOVA; n=3.

3.2.6 Incorporation of FGF-2 and GDNF to starPEG-heparin hydrogels decides on survival/proliferation of primary fetal mesencephalic cells

Since the influence of matrix elasticity and biomolecular functionalization of starPEG-heparin hydrogels on cellular processes could be demonstrated, the influence of other hydrogel-bound growth factors on cell survival and cell fate has to be investigated. Cardinally GDNF counts as promising factor for the treatment of PD. Therefore, RGD-modified hydrogels were loaded either with GDNF, FGF-2 or with a combination of both factors in a concentration of 1 µg/ml for each factor. Studies were focused on the influence of soft hydrogels with a low star-PEG heparin ratio (γ=2) due their possible use in cell transplantation strategies. Primary fetal mesencephalic cells were isolated at E14, seeded with a density of 2,500 cells per mm^2 on biohybrid gels and were cultured for 7 days.

Figure 14 displays survival of primary fetal mesencephalic cells on hydrogels with lowest cross-linking degree (γ=2). Bright field images show the different cell survival on hydrogels with different biomolecular functionalization after 5 DIV (Figure 14A). Like already investigated, a high cell survival was observed on RGD+FGF-2-bound hydrogels with nearly a homogenous cell culture. In contrast, on RGD-modified hydrogels loaded with GDNF cells grew in aggregates and with considerably lower cell survival. The combination of both growth factors with RGD-bound gels seems to exhibit similar survival rates like for RGD+FGF-2-loaded hydrogels.

These observations were confirmed by quantitative analysis of Hoechst 33342^+ cells indicating the total cell number per mm^2 after 7 DIV (Figure 14C). A similar cell survival on FGF-2 loaded hydrogels (5885±112 cells/mm^2) and on hydrogels with both growth factors (5322±691 cells/mm^2; F) were observed. In contrast to that, only very few cells were investigated on RGD-modified hydrogels loaded with GDNF (183±53 cells/mm^2; P<0.005, Student's t-test; n=3).

To identify the amount of dead cells under these conditions after 7 DIV cells were stained for PI, a commonly used agent identifying dead cells, and cell nuclei were counterstained with Hoechst 33342. Figure 14B shows representative fluorescence images of Hoechst/PI staining confirming the observations for cell survival rates obtained from bright field images.

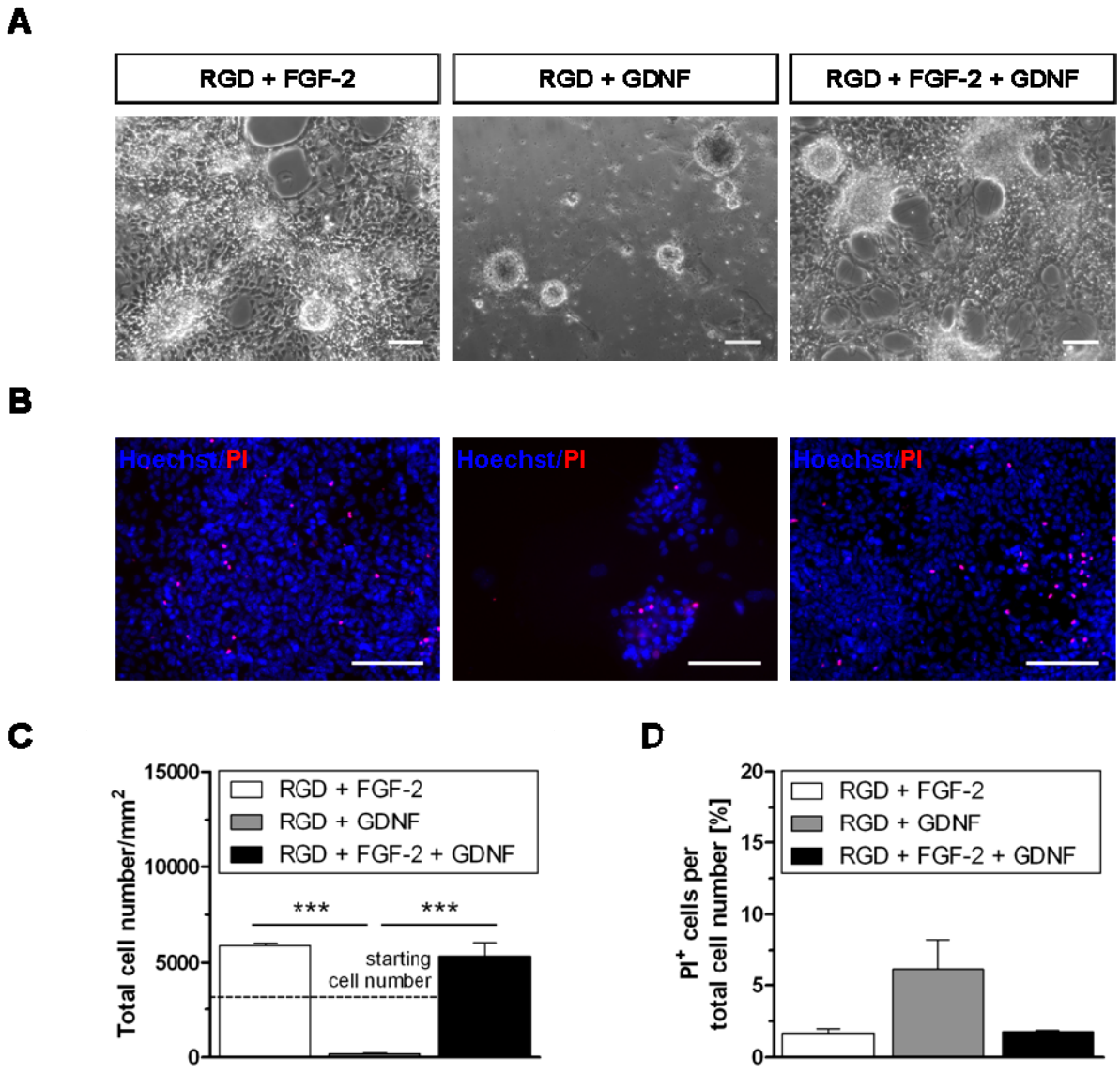

Figure 14: Survival of primary fetal mesencephalic cells on starPEG-heparin hydrogels with different biomolecular functionalizations.
Cells were isolated and seeded at a density of 2,500 cells per mm^2 on hydrogels with different modifications. (A) Representative bright field images show different survival of primary fetal mesencephalic cells on hydrogels after 5 DIV. (B) Representative fluorescence images were observed with staining for Hoechst 33342 and PI after 7 DIV. Scale bar, 100 µm. (C) The survival of cells was analyzed by quantification of Hoechst$^+$ cells after 7 DIV. (D) The percentage of dead cells (PI$^+$) is plotted in respect to culture conditions. Scale bar, 100 µm. Data are expressed as mean ± S.E.M. *** - *P*<0.005, Student's t-test; n=3.

In Figure 14D the quantified number of PI$^+$ cells as percentage to the total cell number is plotted with respect to hydrogel modifications. Generally, only low rates (< 10%) of apoptosis and/or necrosis were observed. No significant differences were observed, but highest values were obtained for cells on GDNF-loaded gels, where 6.2±2.1% of the cells were PI positive. Hydrogels with FGF-2 (1.7±0.3%) and the

combination of FGF-2 and GDNF (1.8±0.1%) showed very low cell death (*P*>0.05, Student's t-test; n=3).

In summary, single GDNF-bound hydrogels does not promote cell survival. This inhibitory effect could be evaded by combining GDNF with FGF-2 in the hydrogel. Furthermore, the amount of dead cells on hydrogels is very low.

To further investigate the influence of FGF2, GDNF and the combination of both factors bound to the hydrogel on survival of other cell types, primary fetal mesencephalic cells were seeded on hydrogels and cultured for 7 DIV. Cells were stained for neural stem cell marker Nestin, for the oligodendrocyte precursor cell marker Olig2, the astroglial cell marker GFAP, the marker for mature neurons MAP2 and Hoechst 33342 to visualize cell nuclei (Figure 15).

Immunofluorescence images show the amount of marker positive cells for different hydrogel modifications (Figure 15A). Cells grown on hydrogels loaded with FGF-2 or the combination of FGF-2 and GDNF showed similar amounts for investigated cell markers, whereas cells on GDNF loaded hydrogels exhibited a slight different marker expression.

The quantitative analysis of the surviving cell types on hydrogels are displayed in Figure 15B. Cells grown on hydrogels loaded with FGF-2, GDNF or the combination of both factors were similarly positive for the neural stem cell marker Nestin (FGF-2: 16±4%; GDNF: 14±7%; FGF-2+GDNF: 13±2%; F-value 0.117, *P*>0.05, ANOVA; n=3). Also the amount of MAP2 positive cells was investigated to be equal for every hydrogel setting (FGF-2: 4.3±0.4%; GDNF: 19±10%; FGF-2+GDNF: 5.8±1.5%; F-value 1.927, *P*>0.05, ANOVA; n=3). Tremendous differences were observed regarding the percentage of $Olig2^+$ and $GFAP^+$ cells grown on hydrogels loaded with different growth factors. A significant increase in $Olig2^+$ cells in respect to total cell number were observed on hydrogels loaded with FGF-2 (27±5%, F-value 17.898, *P*<0.005, ANOVA; n=3) and FGF-2+GDNF (16±3%, F-value 17.898, *P*<0.005, ANOVA; n=3) compared to GDNF loaded hydrogels (0.6±0.4%, n=3). In contrast to that, a significant higher proportion of cells were $GFAP^+$ on GDNF loaded hydrogels (9.4±1.9%; F-value 17.107, *P*<0.01, ANOVA; n=3) compared to cells on FGF-2 and FGF-2+GDNF loaded hydrogels, respectively (FGF-2: 0.9±0.1%; FGF-2+GDNF: 1.6±0.4%, n=3).

Taken together, these findings demonstrate that the survival of distinct cell types is dependent on the growth factor which is loaded to the hydrogel.

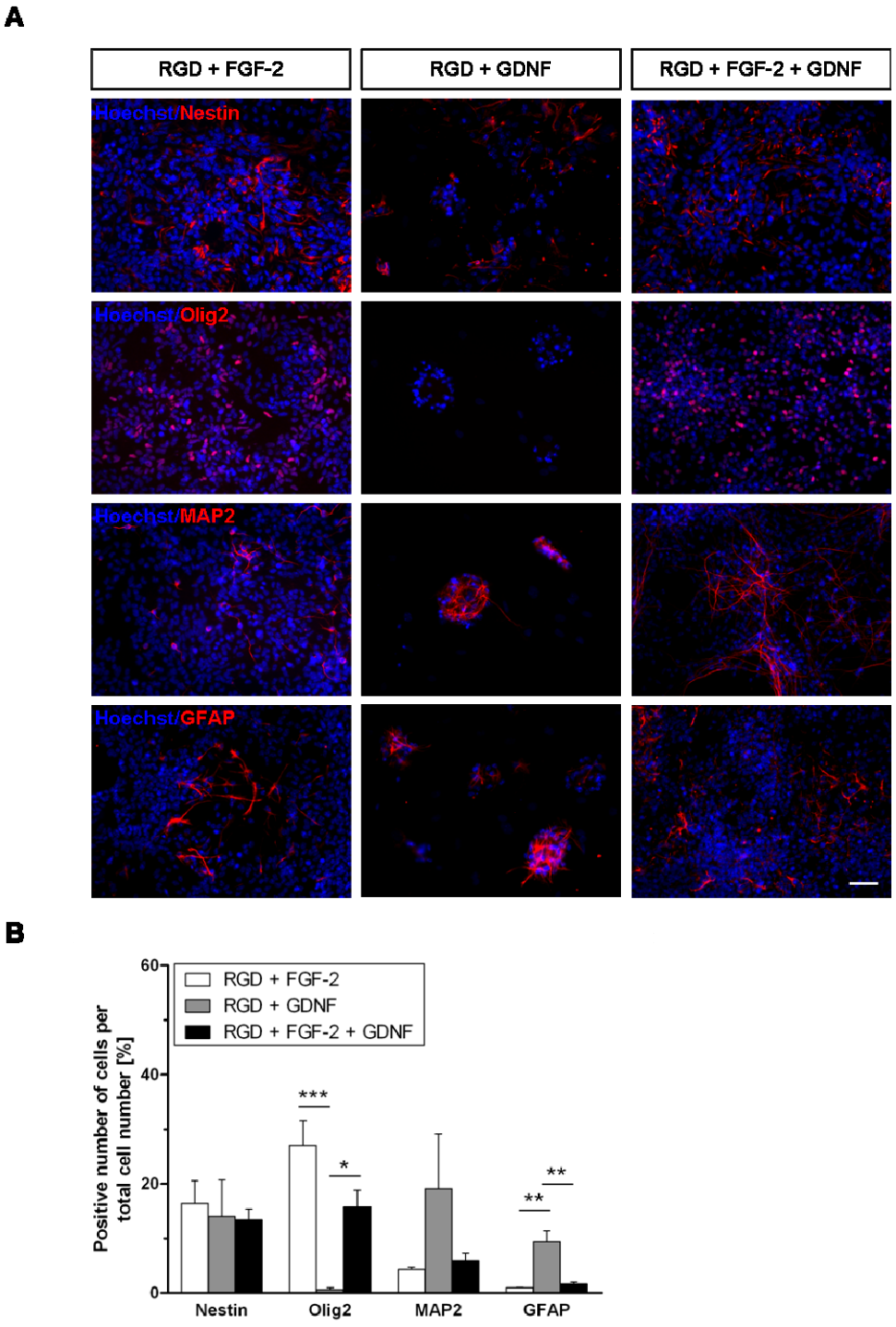

Figure 15: Influence of FGF-2 and GDNF loaded hydrogels on survival of different cell types of primary fetal mesencephalic cells.
Cells were isolated and seeded at a density of 2,500 cells per mm^2 on hydrogels with different modifications. (A) Representative fluorescence images were observed with staining for Nestin, Olig2, MAP2, GFAP or Hoechst 33342 after 7 DIV. (B) Quantitative analysis of cell types in respect to total cell number. Scale bar, 50 µm. Data are expressed as mean ± S.E.M. * - *P*<0.05, ** - *P*<0.01, *** - *P*<0.005, ANOVA; n=3.

3.2.7 Fetal primary mesencephalic cells express MMP2 and migrate into cleavable starPEG-heparin hydrogels

After studying interactions of midbrain-derived cells with the biohybrid hydrogel in two-dimensional cell culture studies, further investigations towards a three-dimensional growth of cells within a biomaterial has to be done in terms of *in vivo* applications. Recently, polymer hydrogels were developed containing cleavable peptide sequences allowing a degradation of the matrices upon enzymatic activity of cells. Matrix metalloproteases (MMPs) are cell-released enzymes attracting special attention due to their importance in cell-mediated ECM remodeling during wound healing and tissue regeneration. Therefore, MMP-cleavable peptide sequences were incorporated into the starPEG-heparin hydrogel system allowing a cell-mediated degradation of the biomaterial. To proof that cells derived from fetal midbrain express MMPs and thus are able to cleave such materials, gene expression analysis as well as immunostainings were performed.

For mRNA analysis, either RNA from primary fetal mesencephalic cells cultivated for 7 days on PDL or laminin or directly isolated RNA from mesencephalic tissue were harvested. Figure 16 shows the relative mRNA levels of MMP2 and MMP9 in mesencephalic tissue and in cells cultured either on PDL or on laminin in respect to the housekeeping gene (hydroxymethylbilane synthase – HMBS). The mRNA expression levels of MMP2 and MMP9 in mesencephalic tissue were found to be very low compared to the HMBS (0.5±0.1 and 0.6±0.1, respectively; n=3). Although a similar mRNA expression of MMP2 could be achieved when cells were cultivated on PDL (1.0±0.2; F-value 4.549, $P>0.05$, ANOVA; n=3) or laminin (1.1±0.1; F-value 4.549, $P>0.05$, ANOVA; n=3), no expression of MMP9 were detectable under culture conditions (F-value 55.358, $P<0.005$, ANOVA; n=3).

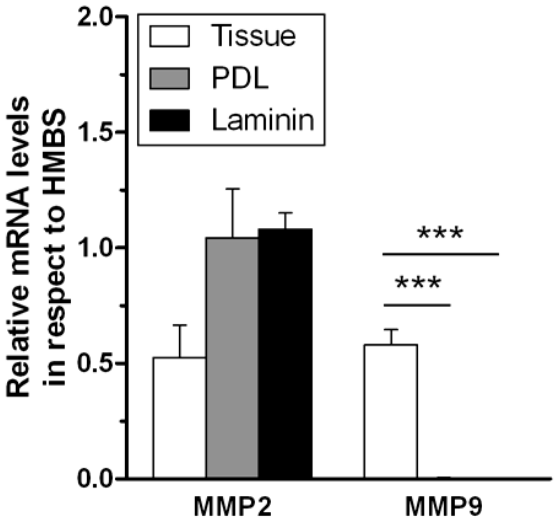

Figure 16: Relative mRNA levels of MMP2 and MMP9 in mesencephalic tissue and *in vitro*.
RNA was isolated either directly from mesencephalic tissue or after cell cultivation for 7 days. Data are plotted in respect to the housekeeping gene HMBS. Data are expressed as mean + S.E.M. *** - $p \leq 0.005$, F-value 55.358, ANOVA; n=3.

To investigate the expression of MMPs on protein level primary fetal mesencephalic cells were cultured for a period of 7 days on PDL and laminin. Later, cells were stained for MMP2 or MMP9 and the marker for dopaminergic cells TH in order to proof their ability to cleave starPEG-heparin hydrogels containing MMP-cleavable peptides. Cell nuclei were counterstained with Hoechst 33342. Figure 17 illustrates the immunostaining for MMP2, the marker for dopaminergic cells TH and Hoechst. Interestingly, not all Hoechst$^+$ cells were also positive for MMP2 in both conditions. Furthermore, a co-labeling of MMP2 with TH could be shown, indicating the ability of dopaminergic cells to produce enzymes degrading ECM or ECM related peptides. For MMP9 no immunostaining either on PDL or on laminin were obtained. Taken together, these investigations on protein level proof the results obtained from mRNA analysis and let assume that cells are able to cleave starPEG-heparin hydrogels containing MMP sequences by the release of MMP2.

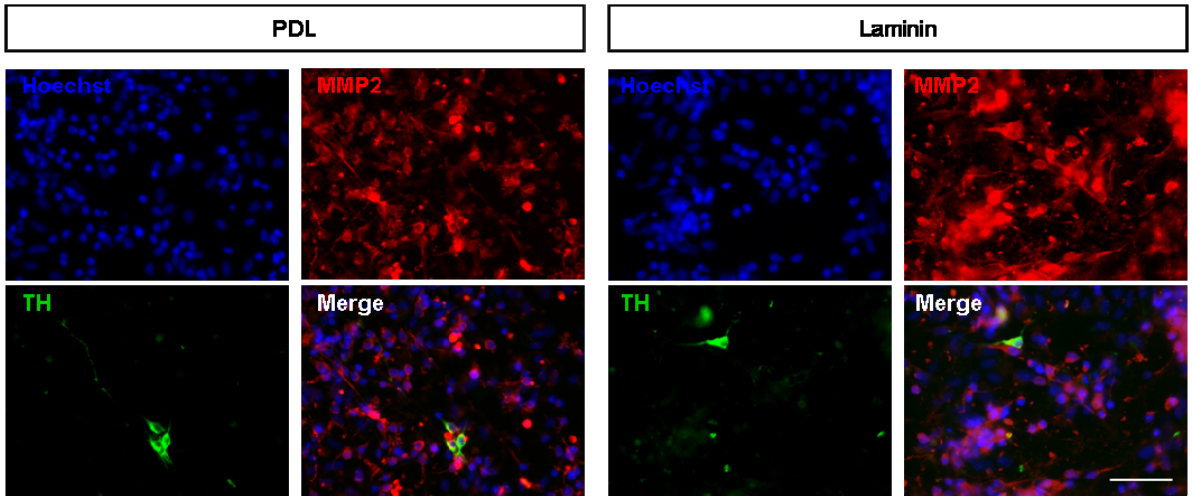

Figure 17: Immunoreaction of primary fetal mesencephalic cells grown on PDL or laminin to the matrix metalloproteinase 2 (MMP2).
Cells were isolated and seeded at a density of 2,500 cells per mm^2 on PDL or laminin. After 7 DIV cells were stained for Hoechst 33342, MMP2 and dopaminergic cells (TH). Scale bar, 50 µm.

To investigate, whether primary fetal mesencephalic cells are able to degrade hydrogels containing MMP-cleavable peptides cells were isolated and seeded with a density of 2,500 cells per mm^2. As comparison the same quantities of cells were also placed on non-cleavable hydrogels. Additionally, the gels were loaded with 1 µg/ml FGF-2 and GDNF. The 3-dimensional migration into hydrogels containing 4 µg of the cell adhesion molecule RGD was analyzed at two different time points (1 DIV, 5 DIV) (Figure 18). After 1 DIV and 5 DIV cells were stained for Hoechst 33342 and phalloidin, a mushroom toxin binding specifically to F-actin. Samples were investigated using confocal laser scanning microscopy performing z-scans. The invasion depth was determined with the software of the microscope.

Figure 18A displays the 3-dimensional migration of phalloidin-stained primary fetal mesencephalic cells after 5 DIV cultured on non-cleavable and cleavable hydrogels. Cells grown on cleavable hydrogels showed a higher migration compared to those cultured on non-cleavable gels.

The measurement of the invasion depth after 1 DIV showed no differences between cells grown either on non-cleavable (19.9±0.5 µm; $P>0.05$, Student's t-test; n=3) or cleavable hydrogels (18.3±1.0 µm; $P>0.05$, Student's t-test; n=3) (Figure 18B). After 5 DIV a significantly higher penetration of cells into hydrogels containing MMP-cleavable sequences (26.9±2.0 µm; $P<0.05$, Student's t-test; n=3) compared to

hydrogels without MMP-cleavable sequences (17.6±0.7 µm; *P*<0.05, Student's t-test; n=3) were obtained.

In these initial studies the ability of primary fetal mesencephalic cells to degrade starPEG-heparin hydrogels containing MMP cleavable sequences were demonstrated. These findings clear the way to perform further studies with MMP cleavable hydrogels in a more advanced three-dimensional cell culture system and for a further use for tissue remodeling *in vivo*.

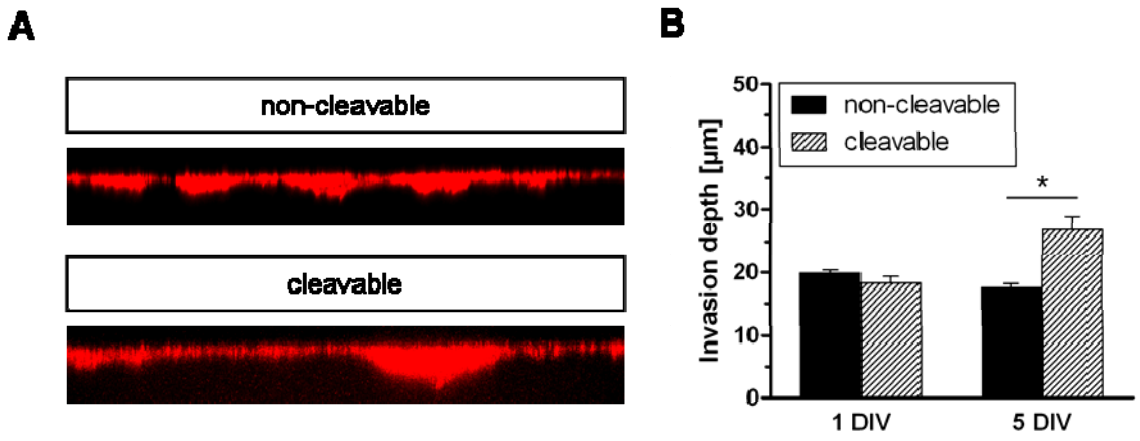

Figure 18: Migration of fetal primary mesencephalic cells grown on cleavable and non-cleavable starPEG-heparin hydrogel.
Cells were isolated and cultured for up to 5 days on cleavable and non-cleavable hydrogels containing. (A) Representative cross-sectional images illustrating the 3-dimensional cell migration of into cleavable and non-cleavable hydrogels containing 4 µg RGD after 5 DIV. (B) After 5 DIV the quantification of the invasion depth illustrates the ability of fetal primary mesencephalic cells to degrade hydrogels containing 4 µg RGD and MMP-cleavable sequences compared to cells grown on non-cleavable hydrogels with the same RGD content. Data are expressed as mean + S.E.M. * - *P*<0.05, Student's t-test; n=3.

4. Discussion

4.1 Cell adhesion mediating peptide RGD promotes neurite extension

As survival, proliferation, differentiation, migration and process extensions of nerve cells are affected by ECM compounds [123, 125, 127-129] their influence on primary DAergic neurons has not been studied yet. Hence, the influence of several ECM components and the cell adhesion mediating peptide RGD on axo-dendritic outgrowth of DAergic neurons were studied in this work. This included the analysis of the number of neurites extending directly from cell soma (primary neurites) indicating a primary branching capability, the number of all neurites indicating a branching capability of a complete neuron, and the length of neurite extension suggesting a possible role in promoting neurite elongation.

Tropocollagen I, a pro-form of collagen I influenced the primary branching capability of DA neurons significantly. 11% more primary neurites were obtained in respect to PDL meaning that tropocollagen enhance the number of neurites originating from the cell soma. Furthermore, fibronectin and the integrin binding sequence RGD boosted the neurite length of DAergic neurons at about 42% and 69%, respectively. So far, promoting effects of fibronectin and RGD on neurite extension was shown for several other nerve cell types [169-172]. Taken into consideration that ECM molecule influences many cellular functions, a cell-matrix interaction via the cell surface receptors integrin could be responsible for the higher branching on tropocollagen I and the promoting effect of the neurite length on fibronectin and RGD [173].

In respect to the aim of the study to design a biomaterial which can promote beside cell survival and differentiation the neurite length of DAergic neurons, the synthetic peptide RGD was subsequently bound to the hydrogel which was used for further studies in this work.

4.2 StarPEG-heparin hydrogels function as an efficient storage and delivery system for FGF-2 and GDNF

Besides serving as cell carrier a biomaterial should be able to deliver factors promoting cell survival, differentiation and axo-dendritic outgrowth for integration of the transplant into the host brain circuitries. Since PEG hydrogels offer a high biocompatibility [154] and are tunable in their mechanical characteristics and biomolecular functionalization [151], they are of major interest for tissue engineering.

Before analyzing the influence of modified starPEG-heparin hydrogels promoting cell survival and axo-dendritic outgrowth, their capability to function as storage and delivery matrix for FGF-2 and GDNF and their combinations was investigated. Uptake and release studies of FGF-2 and GDNF by the starPEG-heparin hydrogel system were analyzed using ELISA. This polymer system utilizes the affinity of growth factors to heparin through electrostatic interaction between the negatively charged *N*- and *O*-sulfated groups of heparin to lysine and arginine residues of FGF-2 and GDNF [148, 149]. The large excess of heparin in the hydrogel and the ability of each heparin molecule to interact with several cytokine molecules, as reported for FGF-2 [174], allow the immobilization of large quantities of growth factors as well as their co-immobilization. In this work similar quantities of growth factors were immobilized independent whether one or even two factors were simultaneously loaded to the hydrogel suggesting that both factors do not influence each other while penetrating into the network.

The amount of immobilized protein showed for single factors and their combinations similar results independent which factor was loaded. Immobilization of lower quantities revealed the expected results for a lower protein amount but different protein amounts were obtained for FGF-2 and GDNF. This may be explained by the instability of FGF-2 [175].

The release of both proteins showed the expected burst release by the starPEG-heparin hydrogel system within the first 24 hours as previously reported [156]. Such burst characteristics can be caused of surface effects [176]. However, within the first 24 hours ~ 95% of the complete released protein amounts were delivered continued by a slow release for the following 6 days. After seven days, nearly similar protein amounts were released for FGF-2 (~ 21%, 53 ng) and GDNF (~ 18%, 47 ng) even though FGF-2 exhibit a higher heparin binding affinity (23 nM)

[177] compared to GDNF (100 nM) [149]. But the larger protein size of GDNF (2x15 kDa) compared to FGF-2 (17 kDa) may hinder the protein to be released by the hydrogel. This may clear a possible higher release of GDNF due to its lower affinity to heparin. Furthermore, the independent delivery of two growth factors simultaneously could be demonstrated. For both proteins similar quantities of protein were released after 7 days independent whether a single factor or in combination was immobilized FGF-2 single: ~ 21% and in combination: ~ 20%; GDNF single: ~ 18% and in combination: ~ 15%. Hereby, it has to mention that an amount of ~ 80% still remains in the hydrogel. In other reports a delivery of ~ 40% of the immobilized GDNF from PLGA microspheres were observed in a comparable time frame [96, 97]. This difference may occur due to the use of a complete different delivery system as well as delivery conditions.

Taken together, the quantities of single released protein were much higher as observed by Zieris A and co-workers [156]. This could be explained by the use of serum-free medium for their release studies. Zieris A and co-workers could also show that the amount of released FGF-2 under serum-free conditions of ~ 1% FGF-2 could be increased to ~ 10% by adding 0.1% BSA to the medium [157]. In this work, medium was used containing 10% FCS leading to a quantified protein amount of ~ 21% FGF-2. The much higher amount of serum proteins in the medium could be a reason for the higher quantities of FGF-2 detected in this work. Furthermore, the release of GDNF from hydrogels in serum and serum-free medium were studied to investigate the amount of released GDNF in case of NSC cultivation, where serum-free medium is applied. Under serum-free conditions ~ 2% GDNF were released compared to ~ 18% in serum medium. These findings are in consistence with the data observed for FGF-2. Possible reasons for the higher protein amount in serum medium could be most likely explained by the reduction of heparin – growth factor interaction and the subsequent replacement of growth factors by serum proteins [178].

To investigate whether the whole amount of protein was detected by the ELISA and thus proof the efficiency of this method, FGF-2 and GDNF were added at a defined concentration in medium containing 10% FCS, similar to the conditions used for analyzing the release profile of the hydrogels. Although the same protein amounts were dissolved in the medium, different quantities of protein were detected

with a 2-fold less efficiency for FGF-2 compared to GDNF. Reasons for that could be that the immobilized protein was not used from the same company like the ELISA kits which show probably different affinities of antibodies to proteins from other companies. Moreover, ELISA kits from different companies had to be used. Finally, as reported for FGF-2 [175] fast protein degradation may take place, too. To filter out all inconsistence, the data were corrected with the factor obtained from the efficiency studies to come closer to the real protein amounts. But still, in this work, slightly higher amounts of released FGF-2 were detected in all conditions which are again explained by a faster degradation of FGF-2 during the ELISA procedure.

4.3 Effects of cross-linking degree and biomolecular functionalization of starPEG-heparin hydrogel on fetal mesencephalic neural stem cells behavior

It has been shown that NSCs respond to mechanical properties, more precisely a higher substrate stiffness supports NSC survival [142, 179, 180]. In concordance to these studies, higher cell survivals of NSCs were obtained in this work for more cross-linked hydrogels with higher substrate stiffness for unmodified and functionalized materials. Furthermore, biomolecular functionalization of hydrogels with RGD, FGF-2 or the combination of both compounds showed different cell survival rates and cell morphologies indicating the existence of different cell types on hydrogel modifications. Additionally, cell attachment and colony outgrowth was only initiated by RGD on hydrogels. This confirms earlier studies on NSCs grown on RGD-modified polymer networks [181, 182]. Immunostainings verified these speculations by revealing more differentiated cells on RGD modified hydrogels, whereas on FGF-2 releasing gels more undifferentiated cells survived. This finding is of great interest in case of future transplantations of FGF-2 loaded hydrogels in combination with NSCs into the brain: The release of FGF-2 may support NSC propagation at the transplantation site by overcoming microenvironmental cues of brain tissue being a strong inducer of differentiation [183, 184]. Moreover, survival/proliferation of NSCs was increased on FGF-2 loaded hydrogels compared to unmodified and RGD-modified hydrogels. For the combination of RGD and FGF-2 a tremendous increase

in cell survival was found. In both cases the crucial effects of released FGF-2 on NSC proliferation was demonstrated [21].

4.4 Biomolecular functionalization of starPEG-heparin hydrogels differentially influences behavior of various cell types in primary mesencephalic cultures

Isolated primary fetal mesencephalic cells exhibit a mixture of stem/progenitor cells and differentiated cells. Cultivation of primary cells under culture conditions using 10% FCS leads to cell differentiation [185], whereas FGF-2 released by hydrogels may have a proliferative effect on remaining stem/progenitor cells [21]. The survival of primary cells grown on FGF-2 loaded hydrogel with highest cross-linking degree is influenced by mechanical properties contrary to NSCs. On this gel type only few cells survive, which is in consistence with a previous report by Saha K and co-workers [179]. Furthermore, primary cell attachment and colony outgrowth were obtained by any functionalization of the hydrogel, whereas NSCs spread only on RGD bound hydrogels. A reason for the diverse cell survival of NSCs and primary cells might be the different expression of integrins, the cell adhesion receptors [186].

The quantitative analysis of the total cell number of fetal primary mesencephalic cells on hydrogels with different cross-linking degrees and biomolecular functionalization revealed only a low cell survival on plain gels due to the absence of any factors promoting cell viability or cell adhesion. Slightly higher cell survival was obtained on RGD modified hydrogels with intermediate cross-linking degree. Immobilization of FGF-2 to hydrogels boosted cell viability ~ 70-fold in average compared to pure gel. This could be explained by the proliferative effect of FGF-2 [21]. Recently, the supportive effect of PEG/collagen polymer immobilized with FGF-2 on neural cells was also demonstrated [187]. Notably, the cell survival was dramatically reduced on hydrogels with highest cross-linking degree and FGF-2 immobilization indicating an inhibitory effect of the material stiffness [179]. With the addition of RGD, these negative effects could be compensated and a similar cell survival compared to lower cross-linked gels was observed. Similar to the total cell number, FGF-2 and RGD+FGF-2 also promote the survival of mature neuronal cells. Only very poor neuronal cell survival was achieved on pure and RGD modified

hydrogels. The investigation of the survival of DAergic cells revealed complete different results. RGD modified hydrogels showed the best cell survival at low and intermediate cross-linking degree. The loading of FGF-2 to the intermediate hydrogel caused even a significant lower survival of dopaminergic cells suggesting no supportive effect of FGF-2. To conclude, the cell survival is massively influenced by synergistic physical and biomolecular cues of the hydrogel.

Furthermore, the effects of chemical/physical characteristics and biomolecular functionalization of the hydrogel on axo-dendritic outgrowth of DAergic cells were studied. The primary branching of DAergic cells seems to be unaffected by physical and biomolecular signals, whereas the total branching is increased on intermediate hydrogels loaded with RGD or FGF-2. Similar to the results obtained for the total branching DAergic cells with long neurites were observed on intermediate hydrogels with RGD or FGF-2 loading. It is to mention that on highly cross-linked hydrogel without any modification and with FGF-2 loading only less DAergic cells survived which were not testable for their axo-dendritic outgrowth. Taken together, similar to the cell survival studies physical/chemical characteristics and biomolecular functionalization of the hydrogels has strong influence on axo-dendritic outgrowth of dopaminergic cells.

Since GDNF is a potent survival factor for DAergic cells (Lin; Tomac; Schatz) this growth factor were immobilized to RGD modified starPEG-heparin hydrogels alone or in combination with FGF-2 and their influence on cell viability were studied compared to RGF+FGF-2 loaded hydrogels. Since isolated primary cells at embryonic stage E14 contained differentiated and undifferentiated cells, the mitogen FGF-2 released by hydrogels may lead to proliferation of stem/progenitor cells [21]. Compared to the initial amount of cells seeded at day 0 an increase of cells of 135% on RGD+FGF-2 loaded and of 113% on RGD+FGF-2+GDNF bound hydrogels were observed. These effects are mainly attributed to the release of FGF-2 from the hydrogels and thus proof the bioactivity of FGF-2 when released from the gel. In contrast to that, GDNF alone did not support cell survival resulting in a 95% decrease of cells compared to the starting cell density of 2,500 cells/mm^2. The analysis of the percentage of dead cells in respect to the total cell number revealed only low quantities of dead cells (~ 2 – 6%) on hydrogel modifications. This led to the conclusion that low cell survival on single GDNF loaded gels is not related to a high

percentage of dead cells. This could rather be a result of low cell adhesion to GDNF modified gels. Since cell replacement therapies are often hampered by a low cell survival, an implantation of FGF-2 and GDNF loaded hydrogel could be an alternative option to improve cell counts after the transplantation process.

To investigate whether biomolecular functionalization with FGF-2, GDNF and the combination of both factors has an effect on survival of different cell types, immunostainings against markers of undifferentiated and differentiated cells were performed. It was found that the percentage of neural stem cells ($Nestin^+$) as well as of mature neuronal cells ($MAP2^+$) were equal in respect to the total cell number in any tested condition. In contrast to that, the survival of $Olig2^+$ and $GFAP^+$ cells seems to be affected by the growth factors which were released by the hydrogels. FGF-2 loading caused 45 times more $Olig2^+$ cells compared to GDNF loading alone, whereas a combined incorporation of FGF-2 and GDNF led to 26 times more $Olig2^+$ cells. The increased amount of oligodendrocyte precursor cells is either attributed to their higher survival or to the proliferative effect of FGF-2 [21]. Contrary results were obtained for the quantity of $GFAP^+$ cells at each hydrogel modification. In comparison to hydrogels loaded with FGF-2 alone and the combination of FGF-2 and GDNF, 10-fold and 6-fold more $GFAP^+$ cells were quantified on GDNF loaded gels, respectively. To conclude, the biomolecular functionalization of the hydrogel with FGF-2 or GDNF decides on the survival of a certain cell type.

4.5 Enzymatically degradable starPEG-heparin hydrogels allow a localized cellular invasion of primary fetal mesencephalic cells

For a three-dimensional reconstitution of tissues or tissue systems such as the central dopaminergic nigro-striatal pathway in the brain, the implanted biomaterial has to offer various physical and biochemical cues to serve as cell carrier releasing factors that support the regenerative process. Moreover, the transplanted cells should be able to degrade and invade the biomaterial and send axons towards the target region. During CNS development and throughout adulthood matrix metalloproteinases (MMPs) play a pivotal role in cell migration, axonal outgrowth, neuronal differentiation and plasticity [110, 188]. The main functions of these proteolytic enzymes are the remodeling and degradation of the ECM. In order to

develop a biodegradable biomaterial, MMP cleavable peptide sequences (GPQG↑↓IWGQ) were included into the starPEG-heparin hydrogel allowing a localized cellular invasion and a possibly elongation of process extensions into the biomaterial.

In this work initial experiments to explore the effects of primary fetal mesencephalic cultures on MMP-degradable starPEG-heparin were performed. Initially, the mRNA expression levels of MMPs in primary fetal mesencephalic cells were analyzed to proof the ability of this cell source to cleave starPEG-heparin hydrogels containing MMP cleavable sequences. Thereby, the main focus was on the investigation of MMP2 and MMP9 expression levels. Among others, MMP2 and MMP9 are expressed during development of the cerebellar cortex [189]. Moreover, recent studies suggested the secretion of large quantities of MMP2 by NSCs [190]. In this work, only low relative mRNA expression levels of MMP2 and MMP9 were found in mesencephalic tissue. Interestingly, after cultivation of primary fetal mesencephalic cells for one week on substrates frequently used in cell culture (PDL, laminin); similar MMP2 expression levels were analyzed, whereas MMP9 was undetectable. A comparable result was reported for the expression of MMP2 and MMP9 in astrocytes [191]. The immunoreactivity for MMP2 was demonstrated for primary mesencephalic cells cultured for one week either on PDL or on laminin. No differences in the immunolabeling of cells in both conditions were observed. Hereby, it has to mention that not all cells showed immunoreactivity for MMP2, but a co-expression with dopaminergic cells could be demonstrated. As expected from mRNA studies no immunoreactivity for MMP9 was detected when primary fetal mesencephalic cells were cultivated on PDL or laminin.

Taken together, these findings suggest the ability of primary fetal mesencephalic cells to express MMP2 and MMP9, but under culture conditions only MMP2 may facilitate the cleavage of starPEG-heparin hydrogels containing MMP cleavable sequences.

To determine the potential of starPEG-heparin hydrogels containing MMP cleavable peptide sequences for an application primary fetal mesencephalic cells were cultured in an initial cell culture study for five days on cleavable and non-cleavable gels containing RGD, FGF-2 and GDNF. In this work a much stronger invasion of primary fetal mesencephalic cells after 5 DIV and thus their ability to

cleave such biomaterials could be demonstrated compared to non-cleavable hydrogels. Recently, in a study by Chwalek K and co-workers a similar migratory behavior of endothelial cells on VEGF immobilized cleavable hydrogels could already be obtained after 2 DIV [168]. This led assume that the MMP expression is much higher in endothelial cells and/or the immobilized growth factor VEGF function as a stronger attractor compared to FGF-2 and GDNF.

5. Conclusion and Outlook

Several important conclusions concerning the development of a biomaterial for reconstructing the central DAergic nigro-striatal pathway in PD could be drawn from this work:

1) Firstly, the results showed for the first time that tropocollagen I, fibronectin and RGD influence the axo-dendritic outgrowth of primary fetal DAergic cells. Among those, RGD is an interesting peptide due to its ability to be bound to several biomaterials to mediate cell adhesion. In this work, a promoting activity on the neurite elongation of primary DAergic cells could be shown. So far, a supportive effect of this peptide on neurite extensions was observed only for other nerve cells [169-172]. Thus, these data suggest that cell-matrix interactions with RGD not only promote cell adhesion but also could exhibit beneficial effects on neurite length of DAergic cells.

2) Secondly, uptake and release experiments of growth factors from the hydrogel system demonstrated their use as efficient storage and delivery system. The release itself is characterized by an initial burst, which may be supportive for the survival of transplanted cells where a major cell loss occurs during the transplantation process. Furthermore, the simultaneous uptake and release of growth factors were demonstrated. This led conclude, that due to the high heparin content of the hydrogel system any soluble factor with heparin-binding motif can be immobilized to and be delivered by the matrix. Notably, a very high amount of growth factors still remain in the hydrogel and is released slowly over time.

3) Thirdly, for tissue engineering approaches in PD using a starPEG-heparin hydrogel system its influence on several cellular processes has to be clarified. Therefore, the effects of different structural/mechanical and biomolecular cues on two standard cell sources for neural transplantation were evaluated. Depending on the material stiffness and the presence of biomolecules the hydrogel showed distinct growth properties of fetal mesencephalic NSCs and

primary fetal mesencephalic cells. This led to the conclusion that for tissue engineering approaches the biomaterial has to be adapted in respect of the used cellular source as well as the properties of the tissue which has to be replaced has to be taken into account.

Furthermore, with the combined incorporation of RGD and the soluble mitogen FGF-2 the cell survival of both cell sources could be tremendously increased. These findings may be explained by the proliferative effect of FGF-2 on both cell sources containing stem/progenitor cells [21]. Since cell replacement therapies for PD are often hampered by a low cell survival [192] and the fact that the brain is a strong inducer for differentiation [183, 184] these results are of quite importance. Moreover, the presence of RGD in the hydrogel led to differentiation of NSCs, whereas on FGF-2 releasing hydrogels more undifferentiated cells survived. These results are in consistence with the fact that ECM compounds stimulate many cellular processes such as differentiation [125, 127, 129] and the known mitogenic action of FGF-2 [21].

The survival and axo-dendritic outgrowth of DAergic cells, which is the cell type got lost in PD, is also affected by the gel properties. RGD or FGF-2 modification of hydrogels showed the best results for DAergic growth but are still not convincing due their relative low boost effect on survival and axo-dendritic growth of DAergic cells in order to reach the striatum, the target region.

Although GDNF is a potent factor for the survival of DAergic neurons [63, 64, 74] the release of this factor by the hydrogel showed only a marginal cell survival as well as a low percentage of dead cells indicating that a slight cell adhesion is responsible for the low amount of cells growing under these conditions. These results may also point out the urgent need of FGF-2 for a higher cell survival combined with GDNF and/or any other promising factors are needed in a starPEG-heparin based approach. Interestingly, the survival of specific cell types can be stimulated by the growth factors which are loaded. Thereby, a high viability of oligodendrocyte precursor cells on FGF-2 loaded hydrogels was predominant. This result may increase the risk for brain cancer where a vast majority of mitotic cells express Olig2 [193]. Furthermore, the tremendous effect of GDNF in combination with FGF-2 on the survival and

axo-dendritic outgrowth of DAergic cells could be demonstrated by a colleague in our lab suggesting a synergistic effect when both factors are released by the hydrogel.

4) Fourthly, for a three-dimensional reconstitution of the central DAergic nigro-striatal pathway in the brain using the “bridging” technology the biomaterial has to biodegradable. The enzymatic degradation of starPEG-heparin hydrogel containing proteolytically cleavable peptide sequences by primary fetal mesencephalic cells could be demonstrated in this work. Furthermore, the expression of MMP2 by primary fetal mesencephalic cells was shown, whereas MMP9 is only expressed in mesencephalic tissue. This indicates that besides other not evaluated proteinases MMP2 led to the cleavage of the hydrogels.

Future research should focus on three-dimensional cell culture studies using starPEG-heparin hydrogels with cleavable peptide sequences. These experiments should be performed in a customer-made cell culture device which was designed according to several requirements in cooperation with Mathias Ulrich from the Department of Device Development and Construction at the Leibniz Institute of Polymer Research Dresden (Figure 19). This two-chamber device is designed to perform three-dimensional cell culture studies. The two chambers can collect 100 µl of volume and are connected with a channel for the placement of the hydrogel. The channel is constructed in three different lengths (2 µm, 3 µm, 4 µm) and two different widths. The usage of place-holders seeded in the chambers allows an unrestricted preparation of the hydrogel in the channels. Furthermore, the device is autoclavable and suitable for microscopy. Placing cells of distinct brain regions, such as *Substantia nigra* and striatum, in the chambers allows studying the three-dimensional cell outgrowth through cleavable starPEG-heparin hydrogels due to released chemoattractant factors by striatal cells. By applying gradients of signaling molecules to the hydrogel their ability to promote axo-dendritic outgrowth of dopaminergic cells in PEG hydrogels could be evaluated. Brain derived neurotrophic factor (BDNF) and sonic hedgehog (Shh) are promising candidates for axon guidance.

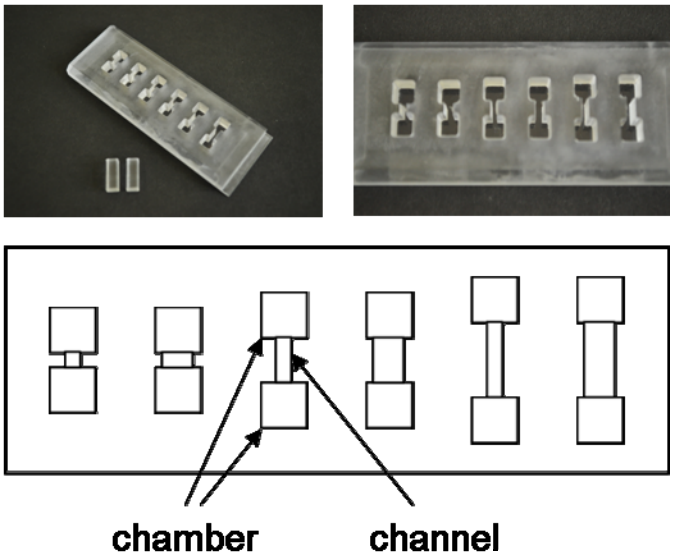

Figure 19: Customer-made cell culture device for three-dimensional cell culture studies.
The two-chambers are connected with a channel available in three different lengths and two different widths.

Furthermore, transplantation of enzymatically degradable starPEG-heparin hydrogels loaded with different growth factors should be performed to proof the *in vivo* applicability of the biohybrid hydrogel to treat PD. This includes stability studies of the transplanted bioscaffolds after remaining several months in the host brain and the investigation of short- and log-term effects to elucidate host-bioscaffold interactions.

6. Abbreviations

aFGF/FGF-1	acidic growth factor
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
CNS	central nervous system
DA	dopamine
DIV	days in vitro
ECM	extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EGF	endothelial growth factor
ELISA	enzyme-linked immunosorbent assay
ES cell	embryonic stem cell
FCS	fetal calve serum
bFGF/FGF-2	basic fibroblast growth factor
GAG	glycosaminoglycan
GDNF	glial cell-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
HMBS	hydroxymethylbilane synthase
IGF-1	insulin-like growth factor
iPS cell	induced pluripotent stem cell
L-DOPA	Levodopa (L-3,4 –dihydroxyphenylalanine)
LV	lentivirus
MAP2	microtubuli-associated protein 2
MMP	matrix metalloproteinases
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridne
NGF	nerve growth factor
NSC	neural stem cells
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
PCR	polymerase chain reaction
PET	positron emission tomography
PEG	poly(ethylene glycol)

PI	propidium iodide
PLGA	poly(lactic-co-glycolic) acid
PFA	paraformaldehyde
POMA	poly(octadecen-*alt*-maleic anhydride)
RCA	Radio Cooperation of America
RGD	Arg-Gly-Asp
SHH	sonic hedgehog
S.E.M.	standard error of the mean
s-NHS	N-hydroxysulfo-succinimid
ß-Tuj1	ß-Tubulin III
6-OHDA	6-hydroxydopamine
TGF-ß	transforming growth factor-beta
TH	tyrosine hydroxylase
TIMP	tissue inhibitors of matrix metalloproteinases

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Meine Person betreffend erkläre ich hiermit, dass keine früheren erfolglosen Promotionsverfahren stattgefunden haben.

Ich erkenne die Promotionsordnung der Fakultät für Mathematik und Naturwissenschaften der Technischen Universität Dresden vom 23. Februar 2011 an.

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